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14. ABSTRACT
The bioactive phospholipids, lysophosphatidic acid (LPA) and phosphatidic acid (PA), regulate pivotal processes related to the pathogenesis of cancer. We characterized a novel lipid kinase, designated acylglycerol kinase (AGK), that phosphorylates monoacylglycerol and diacylglycerol to form LPA and PA, respectively. Confocal microscopy and subcellular fractionation suggest that AGK is localized to the mitochondria. AGK expression was up-regulated in prostate cancers compared with normal prostate tissues from the same patient. Expression of AGK in PC-3 prostate cancer cells markedly increased formation and secretion of LPA. This increase resulted in concomitant transactivation of the EGF receptor and sustained activation of extracellular signal related kinase (ERK) 1/2, culminating in enhanced cell proliferation. AGK expression also increased migratory responses. Conversely, down-regulating expression of endogenous AGK inhibited EGF- but not LPA-induced ERK1/2 activation and progression through the S phase of the cell cycle. Hence, AGK can amplify EGF signaling pathways and may play an important role in the pathophysiology of prostate cancer. Therefore, targeting this kinase, offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

15. SUBJECT TERMS
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INTRODUCTION

Originally known for its pedestrian role as an intermediate in intracellular lipid metabolism, LPA is now recognized as a potent lipid mediator that evokes growth factor-like responses and regulates an array of cellular processes related to pathogenesis of cancer (1). These include stimulating proliferation by increasing cell cycle progression, enhancing cell survival, stimulating motility and inducing tumor cell invasion, and regulating neovascularization (1). There are three established LPA receptors, LPA_1, LPA_2, and LPA_3 (1-3), which are differentially expressed, coupled to a variety of G proteins, and thus regulate diverse cellular responses (1,3). Intriguingly, expression of LPA receptors correlates with more advanced prostate cancer cell lines (4) and LPA_2 and LPA_3 are aberrantly expressed in ovarian cancer cells (5,6), indicating a potential role in the pathophysiology of cancer. Moreover, LPA has a novel intracellular function as a high-affinity ligand for peroxisome proliferators-activated receptor-gamma, a transcription factor that regulates genes controlling energy metabolism (7) and can exacerbate mammary gland tumor development (8).

In addition to actions through conventional GPCR signaling pathways, LPA receptors can indirectly regulate cell functions by transactivating the EGF tyrosine kinase receptor (9-11). This cross-communication between different signaling systems is not only important for the growth promoting activity of LPA (9,11), it also may be a clue to its pathophysiological role in prostate cancer (10), head and neck squamous cell carcinoma (12), and kidney and bladder cancer (13).

LPA is not only an active component of serum, it also accumulates to high concentrations in malignant effusions (14) and has been proposed to be a marker and mediator of ovarian cancer progression (5,6). Although LPA, formed by acylation of glycerol 3-phosphate, is considered to be a key intermediate in *de novo* glycerolipid synthesis, abundant evidence now indicates that bioactive LPA can also be generated by other pathways. LPA is produced from phosphatidic acid

(PA) in activated platelets and ovarian and prostate cancer cells by phospholipase D and subsequent deacylation by phospholipase A_1 or A_2 (15,16). The recent discovery that LPA is generated in the extracellular milieu from lysophosphatidylcholine by the ecto-enzyme autotaxin, known to be involved in tumor invasion, neovascularization and metastasis (17), further supports the notion that LPA is an important regulator of tumor progression (1,18).

Yet another potential pathway for synthesis of LPA is the phosphorylation of monoacylglycerol by a specific lipid kinase (19), an enzyme that has remained an enigma for more than 40 years. We have now cloned and characterized a novel acylglycerol kinase (AGK) that phosphorylates both diacylglycerol to produce PA and monoacylglycerol to form LPA, which in turn activates the EGF receptor, amplifying mitogenic and survival signals and regulating EGF-directed motility (Appendix 1).

BODY

Cloning and characterization of a novel human lipid kinase

In this report, we described the cloning of a lipid kinase that phosphorylates monoacylglycerols to form LPA and demonstrated that it is highly expressed in prostate cancers. We also describe characterization of its enzymatic activity, localization, and signaling pathways that it modulates.

AGK expression enhances cell growth through LPA receptors

Growth promotion is one of the most prominent effects mediated by LPA (1). Consistent with its ability to increase LPA synthesis, transient or stable expression of AGK enhanced proliferation of diverse cell types, including PC-3 cells (Fig. 4C-E, Appendix 1) and NIH 3T3 fibroblasts. The growth promoting effect of AGK was observed even in the presence of sub-optimal serum concentrations (Fig. 4C, Appendix 1). Although AGK stimulates growth, it had no cytoprotective effects on apoptosis induced by serum deprivation or the anti-cancer drug

doxorubicin. As expected, exogenous LPA increased proliferation of both AGK and vector transfectants to the same extent (Fig. 4D Appendix 1). Addition of MOG, to cells cultured in serum-free medium had a minimal effect on vector transfectants and significantly stimulated proliferation of AGK-expressing PC-3 cells (Fig. 4E Appendix 1). This is probably due to rapid metabolism and degradation of MOG and thus, only in AGK expressing cells is sufficient LPA produced and secreted (Fig. 3F Appendix 1) to stimulate proliferation.

The growth promoting effects of AGK were also examined by DNA flow cytometry. FACS analysis revealed that 83 ± 0.4 % of the vector transfectants were in Go/G1 phase and 7 ± 0.04 % and 10 ± 0.4% were in S and G2/M phases, respectively. Overexpression of AGK reduced the fraction of cells in Go/G1 to 73 ± 0.1% and increased the proportion in the S phase by more than 2-fold (15 ± 0.1 %), without significantly affecting the proportion in the G2/M phase (12 ± 0.1 %).

Although it is well established that the mitogenic effects of LPA in many cell types are mediated by binding to its specific GPCRs (1), intracellular actions have also been suggested (20), possibly as an agonist of the nuclear transcription factor PPARgamma (21). To examine the potential involvement of intracellular actions of LPA generated by expression of AGK, its mitogenic effects were determined in rat hepatoma RH7777 cells that do not express $LPA_{1\text{-}4}$ and do not respond to LPA (22). However, in contrast to PC-3 and NIH 3T3 cells, RH7777 cells did not show an increase in DNA synthesis in response to expression of AGK as measured by incorporation of BrdU into nascent DNA (Fig. 4F Appendix 1). Moreover, although GW9662, a selective antagonist of PPARg, inhibited proliferation of vector transfected PC-3 cells, it did not abrogate the mitogenic effect of AGK (Fig. 5A Appendix 1). Consistent with previous results (23,24), we found that LPA_1, LPA_2, and LPA_3 are expressed in PC-3 cells. It is known that LPA_1 couples to pertussis toxin (PTX)-sensitive Gi, whereas LPA_2 and LPA_3 couple also to Gq. In PC-

3 cells, LPA-regulated mitogenic signaling is mediated by Gbg subunits derived from PTX-sensitive Gi proteins (23,25). In agreement, PTX pretreatment not only inhibited growth of vector transfectants, it also markedly decreased the growth promoting effects of AGK (Fig. 5A Appendix 1).

AGK-induced ERK1/2 activation requires EGFR

Previously, it has been suggested that EGFR activation is required for signal relay from LPA receptors to ERK1/2 activaton in prostate cancer cells (10,24,26,27). AGK expression markedly increased activation of ERK1/2, as determined with a phospho-specific antibody, which was further enhanced by serum (Fig. 5B) and EGF (Fig. 5D and Fig. S1C Appendix 1). To further confirm that activation of the EGFR was necessary for AGK-stimulated ERK activation, we utilized the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478. As expected, AG1478 abolished EGFR-induced tyrosine phosphorylation. AG1478 blocked AGK-mediated ERK1/2 phosphorylation (Fig. 5D Appendix 1) and decreased its mitogenic effect (Fig. 5E Appendix 1) and also inhibited MOG-stimulated proliferation by 35 ± 4%. Nonetheless, prolonged treatment with AG1478 did not affect AGK protein levels (Fig. 5E, insert Appendix 1).

Taken together, these results suggest that the tyrosine kinase activity of EGFR contributes to AGK-induced activation of the ERK cascade and that stimulation of DNA synthesis is mediated not only by transactivation of EGFR, but also by direct activation of Gi-coupled LPA receptors.

Involvement of AGK in motility

Transactivation of the EGFR has also been implicated in motility of cancer cells (12). In agreement, AGK overexpression enhanced migration of PC-3 cells towards EGF, which was

blocked by the EGFR inhibitor AG1478 (Fig. 6A Appendix 1). AGK also enhanced migration of NIH 3T3 fibroblasts towards serum.

In the Boyden chamber cell migration assay, differences in cell shape and size may affect passage through the pores in the membrane but do not affect the *in vitro* wound closure assay. AGK expression also enhanced closure of the wounded area, especially in the presence of EGF and the AGK substrate MOG (Fig. 6B,C Appendix 1). In contrast, wound closure induced by LPA was not affected by AGK expression. AGK-induced wound closure was also blocked by AG1478, supporting a role for EGFR transactivation in AGK-induced migratory responses.

Involvement of endogenous AGK in cell cycle progression

Surprisingly, we noticed that serum and EGF induced significant increases in AGK expression as determined by quantitative real-time PCR (Fig. 7A Appendix 1). It has previously been shown that stimulated PC-3 cells produce high levels of LPA and also respond to LPA (16). Moreover, in some cancer cells, LPA itself is sufficient to increase its own production, indicating the presence of an autocrine network (28). Consistent with an autocrine function for LPA, we found that LPA also increased expression of AGK by 3 fold in naïve PC-3 cells (Fig. 7A Appendix 1). Since AGK expression was stimulated by these potent growth factors, it was of interest to examine the physiological function of AGK by knocking down its expression with small interfering RNA (siRNA). siAGK, but not control siRNA, markedly reduced AGK mRNA in PC-3 cells, as determined by quantitative real-time PCR, without influencing expression of SphK1 (Fig. 7B Appendix 1). Consistent with its role in synthesis of LPA and PA, the most striking effect of downregulating AGK was reduction of mitochondrial PA and LPA by approximately 30% (Fig. 7C Appendix 1). siAGK completely blocked stimulation of ERK induced by EGF (Fig. 7D Appendix 1) and also markedly reduced migration towards EGF (Fig. 7E Appendix 1). siAGK not only inhibited basal secretion of IL-8, it also reduced secretion of

IL-8 induced by EGF and LPA (Fig. 7F Appendix 1). This was due to specific downregulation of AGK as treatment with control siRNA did not influence IL-8 secretion.

We next examined the role of endogenous AGK in cell growth regulation. Remarkably, treatment with siRNA targeted to AGK markedly decreased DNA synthesis as measured by incorporation of BrdU into nascent DNA (Fig. 8A,B Appendix 1). In contrast, treatment with non-specific siRNA did not alter BrdU incorporation. In agreement, cell cycle analysis revealed that after one day in serum free medium, more than 75% of PC-3 cells transfected with non-specific RNA were in G_o/G_1 phase and only a small fraction were in S and G_2/M phases, which was similar to untreated cells (Fig. 8C Appendix 1). Transfection with siAGK increased the fraction of cells in G_o/G_1 and decreased the proportion in the S phase and, to a lesser extent, in the G_2/M phase. Even in the presence of 10% serum, which markedly increased the proportion of cells in the S phase and G_2/M phase, siAGK, but not control siRNA, further reduced cells in S phase (Fig. 8C Appendix 1).

KEY RESEARCH ACCOMPLISHMENTS

- Cloned an acyglycerol kinase, AGK
- Established the substrates for this kinase and cellular localization
- Showed that AGK expression enhances cell growth through LPA receptors
- Showed that AGK expression enhances EGF-directed motility
- Found that AGK expression promotes IL-8 secretion
- Demonstrated that AGK is involved in transactivation of EGFR
- Downregulation of AGK substantiated its physiological function
- Confirmed that endogenous AGK regulates cell cycle progression
- Discovered that endogenous AGK is critical for EGF-induced ERK1/2 activation

REPORTABLE OUTCOMES

1. Bektas, M., Payne, S. G., Liu, H., Goparaju, S., Milstien, S., and Spiegel, S. 2005. A novel acylglycerol kinase that produces lysophosphatidic acid modulates cross talk with EGFR in prostate cancer cells. J. Cell Biol. 169, 801-811 (Appendix 1)

2. Spiegel, S. and Milstien, S. 2005. Critical role of acylglycerol kinase in epidermal growth factor-induced mitogenesis of prostate cancer cells. Biochem. Soc. Trans. 33:1362-1365 (Appendix 2)

3. Bektas, M. and **S. Spiegel**. 2004. Glycosphingolipids and cell death. Glycoconj. J. 20:39-47. (Appendix 3)

4. Payne, S.G., Milstien, S., Barbour, S.E., and Spiegel, S. 2004. Modulation of adaptive immune responses by sphingosine-1-phosphate. Semin. Cell Devel. Biol. 15:521-527 (Appendix 4)

5. Watterson, K., H. Sankala, S. Milstien, and **S. Spiegel.** 2003. Pleiotropic actions of sphingosine-1-phosphate. Prog. Lipid Res. 42:344-357. (Appendix 5)

6. **Spiegel, S**. and S. Milstien. 2003. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. Biochem. Soc. Trans. 31:1216-1219. (Appendix 6)

7. **Spiegel, S**. and S. Milstien. 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nature Rev. Mol. Cell Biol. 4:397-407. (Appendix 7)

8. Maceyka, M. and **S. Spiegel**. 2003. Sphingosine-1-Phosphate Receptors. Handbook of Cell Signaling, Vol. 2, Ch. 163, pp. 247-251, Elsevier Science. (Appendix 8)

Presentations

1. Sphingosine kinase family: novel functions, Bioscience 2005 – From Genes to Systems, Glasgow, Scotland, July 17-21, 2005.

2. Sphingosine kinase family in cancer cell signaling, French Society of Cell Biology, Symposium on Lipid Functions in Membrane Dynamics, Paris, France, November 7-8, 2005.

3. A novel acylglycerol kinase that produces lysophosphatidic acid modulates crosstalk of growth signals in prostate cancer cells. Bektas, M., SMBL, VCU, July, 2004.

4. Lysophospholipids in Cancer. June 2003. Massey Cancer Center Research Retreat, MCV, Richmond, VA

CONCLUSIONS

In this work, we have cloned the long searched for monoacylglycerol kinase (AGK), a novel type of diacylglycerol kinase which phosphorylates monoacylglycerols, to form LPA. LPA has long been implicated as an autocrine and paracrine growth stimulatory factor for prostate cancer cells. The identification of this novel lipid kinase that regulates its production could provide new and useful targets for preventive or therapeutic measures. Expression of AGK in PC-3 prostate cancer cells markedly increased formation and secretion of LPA. This resulted in concomitant transactivation of the EGF receptor and sustained activation of ERK1/2, culminating in enhanced cell proliferation. AGK expression also increased migratory responses and stimulated secretion of IL-8. Conversely, downregulating expression of endogenous AGK inhibited progression through the S phase of the cell cycle and reduced IL-8 secretion. Hence, AGK can amplify EGF signaling pathways and may play an important role in the pathophysiology of prostate cancer. Because of the well-known role of the EGF receptor in androgen-refractory metastatic prostate cancer, the pathophysiological significance of our novel lipid kinase may be to produce LPA, which in turn can stimulate the release of mature EGF, and thus activate the EGF receptor, amplifying mitogenic and survival signals. Therefore, targeting this kinase that is upstream of the EGF receptor offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

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A novel acylglycerol kinase that produces lysophosphatidic acid modulates cross talk with EGFR in prostate cancer cells

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The bioactive phospholipids, lysophosphatidic acid (LPA) and phosphatidic acid (PA), regulate pivotal processes related to the pathogenesis of cancer. Here, we report characterization of a novel lipid kinase, designated acylglycerol kinase (AGK), that phosphorylates monoacylglycerol and diacylglycerol to form LPA and PA, respectively. Confocal microscopy and subcellular fractionation suggest that AGK is localized to the mitochondria. AGK expression was up-regulated in prostate cancers compared with normal prostate tissues from the same patient. Expression of AGK in PC-3 prostate cancer cells markedly increased formation and secretion of LPA. This increase resulted in concomitant transactivation of the EGF receptor and sustained activation of extracellular signal related kinase (ERK) 1/2, culminating in enhanced cell proliferation. AGK expression also increased migratory responses. Conversely, down-regulating expression of endogenous AGK inhibited EGF- but not LPA-induced ERK1/2 activation and progression through the S phase of the cell cycle. Hence, AGK can amplify EGF signaling pathways and may play an important role in the pathophysiology of prostate cancer.

Introduction

Originally known for its pedestrian role as an intermediate in intracellular lipid metabolism, lysophosphatidic acid (LPA) is now recognized as a potent lipid mediator that evokes growth factor–like responses and regulates an array of cellular processes related to pathogenesis of cancer (Mills and Moolenaar, 2003). Progress in understanding LPA actions has accelerated with the discovery that it is a ligand of several G protein–coupled receptors (GPCRs), termed LPA_1, LPA_2, and LPA_3 (Mills and Moolenaar, 2003). Intriguingly, expression of LPA receptors correlates with more advanced prostate cancer cell lines (Gibbs et al., 2000). In addition to actions through conventional GPCR signaling pathways, LPA receptors can indirectly regulate cell functions by transactivating the EGF tyrosine kinase receptor (Prenzel et al., 1999). This cross-communication between different signaling systems is not only important for the growth-promoting activity of LPA (Prenzel et al., 1999) but it may also be a clue to its pathophysiological role in prostate cancer (Prenzel et al., 1999). The recent discovery that LPA can be generated in the extracellular milieu from lysophosphatidylcholine by the ectoenzyme autotaxin, known to be involved in tumor invasion, neovascularization, and metastasis (Umezu-Goto et al., 2002), further supports the notion that LPA is an important regulator of tumor progression (Mills and Moolenaar, 2003).

A potential pathway for synthesis of LPA is the phosphorylation of monoacylglycerol by a specific lipid kinase (Pieringer and Hokin, 1962), an enzyme that has remained an enigma for more than 40 yr. We have now cloned and characterized a novel acylglycerol kinase (AGK) that phosphorylates both monoacylglycerol to form LPA and diacylglycerol to produce phosphatidic acid (PA), another potent lipid second messenger that mediates mitogenic activation of mTOR (mammalian target of rapamycin) signaling (Fang et al., 2001). LPA produced by AGK in turn activates the EGF receptor, amplifying mitogenic and survival signals and regulating EGF-directed motility. Our results suggest that AGK, which is highly expressed in prostate cancers, might be important for the initiation and progression of prostate cancer, processes in which LPA plays prominent roles (Prenzel et al., 1999; Kue and Daaka, 2000; Kue et al., 2002; Xie et al., 2002; Mills and Moolenaar, 2003).

M. Bektas and S.G. Payne contributed equally to this paper.

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Abbreviations used in this paper: AGK, acylglycerol kinase; DAG, 1,2-dioleoyl-*sn*-glycerol; DAGK, DAG kinase; ERK, extracellular signal related kinase; GPCR, G protein–coupled receptor; LPA, lysophosphatidic acid; MAGK, monoacylglycerol kinase; MOG, 1-oleoyl-2-*sn*-glycerol; PA, phosphatidic acid; PPARγ, peroxisome proliferator-activated receptor-γ; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; SphK, sphingosine kinase.

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http://www.jcb.org/cgi/content/full/jcb.200407123/DC1

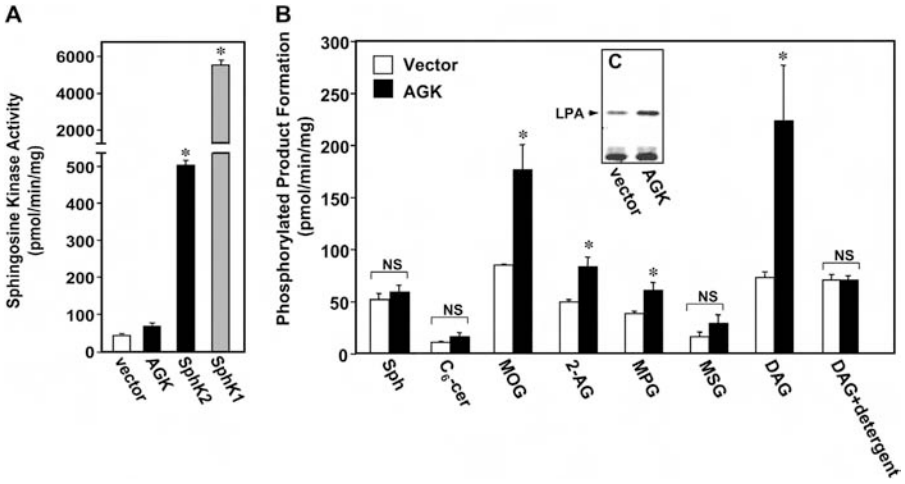


Figure 1. **Lipid kinase activity of recombinant AGK.** (A) NIH 3T3 cells were transiently transfected with vector, hSphK1, hSphK2, or hAGK. After 24 h, cells were lysed and sphingosine-phosphorylating activity in cell lysates was measured with 50 μM D-*erythro*-sphingosine (Sph) substrate added as a BSA complex as described previously (Liu et al., 2000). (B) Lipid-phosphorylating activity was determined in cell lysates from NIH 3T3 cells transiently transfected with vector (open bars) or hAGK (closed bars). The following lipids were tested: Sph, C_6-cer; 1-oleoyl-2-*sn*-glycerol (18:1), MOG; 2-arachidonoyl-glycerol (20:4), 2-AG; 1-palmitoyl-2-*sn*-glycerol (16:0), MPG; 1-stearoyl-2-*sn*-glycerol (18:0), MSG; or diacylglycerol (1,2-dioleoyl-*sn*-glycerol), DAG. Where indicated, octyl-β-glucopyranoside detergent was added to a final concentration of 1.5%. The data are expressed as picomoles of phosphorylated product formed per minute per milligram ± SD. Similar results were obtained in four additional experiments. *, $P < 0.05$ by *t* test. (C) TLC separation of products formed with MOG as substrate visualized with a phosphoimager.

Results

A new lipid kinase catalyzes the phosphorylation of acylglycerols to generate LPA and PA

While searching for additional isoforms of sphingosine kinase (SphK), the enzyme that catalyzes the formation of sphingosine-1-phosphate (S1P), another serum-borne lysophospholipid structurally similar to LPA, we cloned a related gene that encodes a 422–amino acid protein (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1). Although this new kinase was cloned based on its homology to SphKs, it only displayed barely detectable phosphorylating activity with sphingosine as substrate when compared with cells transfected with SphK1 or SphK2 (Fig. 1 A). Moreover, there were no detectable changes in the levels of the sphingolipid metabolites, ceramide, sphingosine, or S1P, in cells overexpressing this lipid kinase. Furthermore, when AGK transfectants were labeled with [^{3}H]sphingosine, there were no significant increases detected in the formation of [^{3}H]S1P compared with vector-transfected cells (unpublished data).

We examined in vitro kinase activity with an array of lipid substrates, including different ceramide species and glycerolipids, such as 1,2-dioleoyl-*sn*-glycerol (DAG), glycerol-3-phosphate, anandamide, phosphatidylinositol, phosphatidylglycerol, cardiolipin, and the monoacylglycerol 1-oleoyl-2-*sn*-glycerol (MOG). Significant phosphorylated products were only detected with monoacylglycerols and diacylglycerols as substrates, but not with any other lipid tested, including ceramide and sphingosine (Fig. 1 B); thus, we have designated this lipid kinase as an AGK. Although AGK contains a DAG kinase (DAGK) catalytic domain (Fig. S1), it did not significantly phosphorylate DAG when activity was measured in the presence of the detergent octyl-β-glucopyranoside (Fig. 1 B), as usually used for DAGK activity measurements (Bunting et al., 1996), suggesting that AGK is distinct from other known DAGKs.

Previously, a partially purified bovine brain monoacylglycerol kinase (MAGK) was reported to prefer substrates containing unsaturated fatty acid esters (Shim et al., 1989; Simpson et al., 1991). Interestingly, AGK has higher activity with substrates containing a C18 fatty acid with one double bond, as monoacylglycerol with an oleoyl (18:1) substitution in the *sn1* position was phosphorylated to a greater extent than 1-palmitoyl-2-*sn*-glycerol (16:0), which was a better substrate than 1-stearoyl-2-*sn*-glycerol (18:0) (Fig. 1 B). Moreover, 1-*sn*-2-arachidonoyl-glycerol, an endogenous cannabinoid receptor ligand (Sugiura et al., 2000), was also a reasonably good substrate (Fig. 1 B). Like the crude bovine brain MAGK activity (Shim et al., 1989), AGK required magnesium for maximal activity, whereas other divalent cations, including Ca^{2+} and Zn^{2+}, inhibited phosphorylation of MOG. Similar to brain MAGK, AGK also had higher activity in the presence of 0.03% deoxycholate, although enzymatic activity was completely abolished by most other detergents, including Triton X-100, Triton X-114, CHAPS, and β-octylglucopyranoside (Fig. 1 B and not depicted).

While this manuscript was in revision, Waggoner et al. (2004) showed that AGK expressed in bacteria phosphorylates DAG as well as MOG and ceramide, but not sphingosine, whereas in lysates of AGK-overexpressing cells, ceramide was not phosphorylated (Fig. 1 B), nor did we detect any phosphorylation of ceramide or sphingosine in vivo.

Subcellular localization of AGK

Confocal immunofluorescence microscopy revealed that AGK was distributed in a punctate, reticular pattern in NIH 3T3 cells (Fig. 2 A), which is reminiscent of a mitochondrial localization. There was no significant colocalization with the ER marker calnexin (Fig. 2 A). On the other hand, AGK expression clearly colocalized with mitochondria stained with MitoTracker red (Fig. 2 A). Similar mitochondrial localization of AGK was also observed in HEK 293 and PC-3 cells (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1), indicating that the subcellular distribution was not cell type specific. In agreement, although AGK does not contain a canonical mitochondrial localization signal, the MitoProt II website predicts an 80% probability of mitochondrial localization,

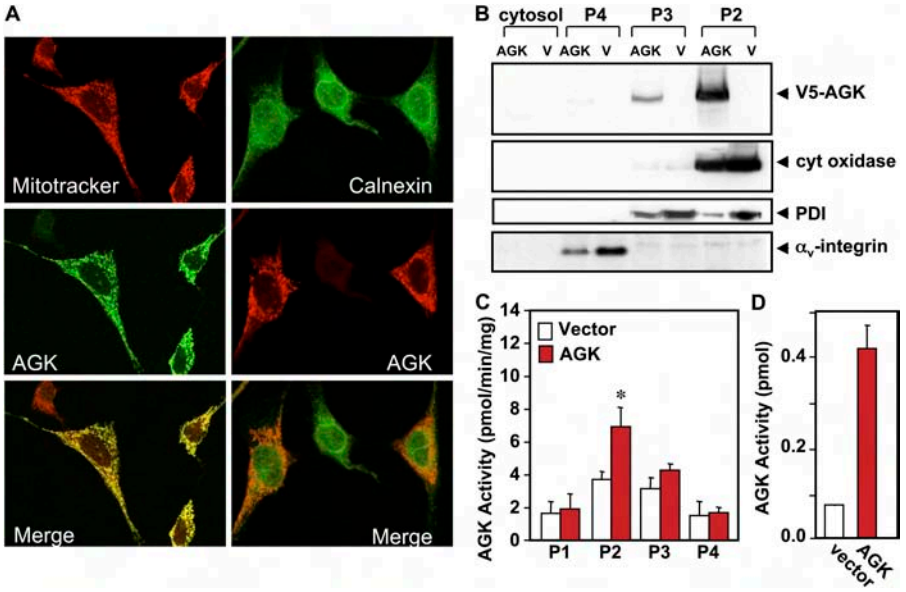


Figure 2. **Subcellular localization of AGK.** (A) NIH 3T3 fibroblasts were transiently transfected with V5-tagged AGK and mitochondria stained with MitoTracker red. The ER was visualized with anti-calnexin antibody followed by FITC-conjugated anti-rabbit as the secondary antibody. AGK was stained with monoclonal anti-V5 antibody followed by secondary FITC-conjugated or Texas red–conjugated anti–mouse antibody. Cells were visualized by dual wavelength confocal microscopy. Superimposed merged pictures are shown in the bottom panels, with yellow indicating colocalization. (B and C) Activity and expression of AGK in subcellular fractions. Lysates from HEK 293 cells transfected with vector or V5-AGK and P2 (mitochondria), P3 (ER and Golgi), P4 (plasma membrane), and cytosol fractions isolated. The P1 fraction containing nuclei and unbroken cells was not examined. 25 μg of proteins were resolved by SDS-PAGE and immunoblotted with anti-V5 antibody or with antibodies to the specific organelle markers anti–cytochrome *c* oxidase, anti-phosphodisulfide isomerase (PDI), and anti-α_v-integrin. AGK activity was also determined in each subcellular fraction with MOG as substrate. Results are means ± SD of triplicate determinations. Similar results were obtained in two additional experiments. *, $P < 0.05$ by *t* test. (D) 400-μg aliquots of lysates from HEK 293 cells transiently transfected with vector (open bars) or V5-AGK (closed bars) were immunoprecipitated with anti-V5 antibody as described in Materials and methods, and AGK activity was determined in the immunoprecipitates. Data are expressed as picomoles of LPA formed in 30 min and are means ± SD of duplicate determinations.

and the program TMpred predicted one transmembrane region from amino acid 11 to 30.

To further substantiate the localization of AGK, protein expression and enzymatic activity were examined in subcellular fractions prepared by differential centrifugation. V5-Epitope–tagged AGK with the predicted MW of 46.4 kD was highly enriched in the P2 mitochondria fraction (Fig. 2 B). Much less AGK was present in the P3 fraction containing intracellular membranes of the ER and Golgi or in the P4 plasma membrane fraction. In concordance with the protein expression pattern, the highest AGK-specific activity was in P2 (Fig. 2 C).

AGK regulates LPA and PA in vivo

To definitively demonstrate that AGK has intrinsic kinase activity rather than affecting the activity of some endogenous lipid kinase, phosphorylation of acylglycerol substrates after specific pull-down of epitope-tagged AGK expressed in HEK 293 cells with V5 antibody was determined. Although the V5 antibody inhibits AGK, phosphorylation of MOG was sixfold greater in immunoprecipitates from V5-AGK transfectants than vector transfectants (Fig. 2 D) and there was no significant phosphorylating activity with other lipid substrates (not depicted).

To identify the phosphorylated lipids produced by AGK in vivo, vector and AGK PC-3 transfectants were incubated with ^{32}P-labeled orthophosphate and labeled phospholipids in isolated mitochondria examined (Fig. 3 A). Expression of AGK resulted in 80% increase of ^{32}P-labeled PA without significantly affecting labeling of the other mitochondrial phospholipids. Because it is known that LPA synthesized in mitochondria can readily exit this organelle (Chakraborty et al., 1999) or be rapidly metabolized to PA, changes in total cellular phospholipids were also examined (Fig. 3, B–D). There were no obvious differences in labeling of the major known cellular phospholipids in AGK-expressing cells compared with the vector cells. However, two-dimensional HPTLC analysis revealed that a labeled phospholipid that comigrated with authentic LPA (Fig. 3, B and C), although barely detectable in vector cells, was increased threefold in AGK-expressing cells. Moreover, this phospholipid was eliminated by treatment with phospholipase B, which hydrolyzes the ester bonds of lysophospholipids, confirming its identity as LPA. Labeled PA was also increased in these transfectants (Fig. 3 D), albeit much less than LPA. Of note, in these cells, AGK mRNA levels relative to 18S RNA were increased by almost twofold over endogenous expression from 1.2 ± 0.1 to 2.3 ± 0.2, as determined by quantitative PCR.

It has previously been shown that cancer cells secrete LPA (Mills and Moolenaar, 2003). Small amounts of labeled lysophospholipids, including LPA, were secreted by vector transfected PC-3 cells. However, secretion of ^{32}P-labeled LPA was significantly increased threefold by overexpression of AGK (Fig. 3, F and G), indicating that AGK increases both intracellular and extracellular levels of LPA. It should be noted that AGK was not detectable in the medium by immunoblotting nor did its expression cause apoptosis of cells, suggesting that appearance of LPA in the media is not a result of cell death.

All members of the DAGK and SphK superfamily have a conserved GDG sequence in the glycine-rich loop of the putative ATP binding region and a single point mutation of the second conserved glycine residue to aspartate has been used to prepare catalytically inactive DAGK (Topham and Prescott, 1999) and SphK (Pitson et al., 2002). Similarly, site-directed mutagenesis of the equivalent residue in AGK (G126E) resulted in a complete loss of phosphorylating activity (Fig. S2 B), and its expression had no discernible effects on ^{32}P-labeled LPA, PA, or other phospholipids (Fig. S2 C). However, like wild-type AGK, this catalytically inactive mutant was localized to the mitochondria (Fig. S2 A)

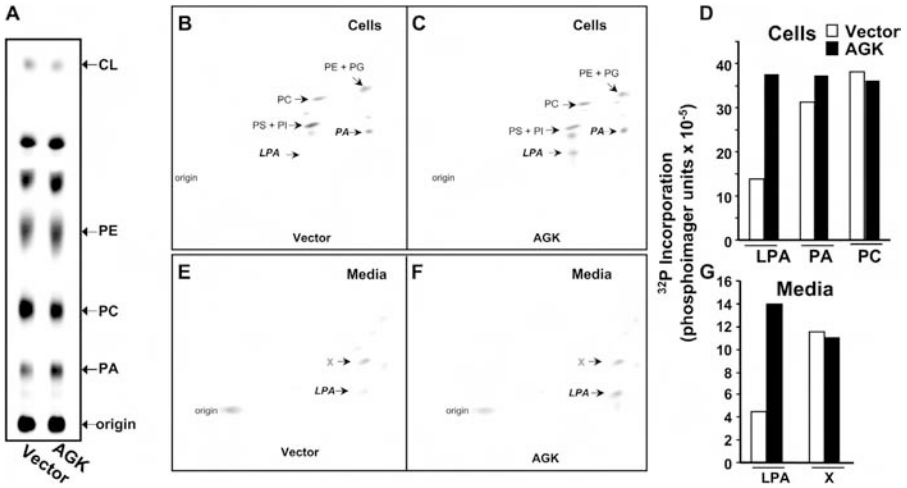


Figure 3. **Effect of AGK on phospholipids.** (A) PC-3 cells stably transfected with vector or AGK were labeled with ^{32}P-orthophosphate for 2 h. Phospholipids were then extracted from mitochondria isolated by differential centrifugation. After separation of equal amounts of ^{32}P-labeled phospholipids by one-dimensional TLC, radioactive spots were visualized with a phosphoimager and the indicated lipids were identified based on comigration with authentic standards. The ratio of ^{32}P-PA to ^{32}P-PC in vector and AGK transfectants was 0.38 ± 0.02 and 0.68 ± 0.03, respectively. (B–G). LPA production and secretion induced by expression of AGK. PC-3 cells stably transfected with vector or AGK were prelabeled with ^{32}P-orthophosphate for 2 h, washed, and incubated for 2 h in chemically defined medium. Lipids were extracted from cells (B–D) and media (E–G). Equal amounts of ^{32}P-phospholipids were separated by two-dimensional HPTLC, first in chloroform/methanol/formic acid/water (60:30:7:3, vol/vol), followed by chloroform/methanol/ammonium hydroxide/water (50:40:8:2, vol/vol). Radioactive spots were visualized with a phosphoimager, and the indicated lipids were identified based on comigration with authentic standards. (D and G) ^{32}P incorporation into the indicated phospholipids (LPA, PA, PC, and unidentified phospholipid [X]) was quantified by phosphoimager. Similar results were obtained in two additional experiments.

AGK is highly expressed in prostate cancer

As LPA has been most prominently associated with growth-promoting effects and probably contributes to cancer (Mills and Moolenaar, 2003), it was of interest to examine the expression of AGK in normal tissues and cancers. By Northern analysis, a 2.6-kb AGK mRNA was widely expressed (Fig. 4 A), most abundantly in heart, kidney, muscle, and brain. Importantly, using a matched human tumor/normal tissue expression array, we found that AGK expression was significantly up-regulated in prostate cancers compared with the normal prostate tissues from the same patient (Fig. 4 B). However, not all tumor tissues show an increase in AGK expression. In uterine, cervical, and stomach cancers, there also appeared to be higher expression of AGK compared with the normal tissues, whereas AGK expression seems to be reduced in colon cancer. AGK was also expressed in many types of human cancer cell lines (Fig. 4 B), including prostate cancer cells, such as androgen-responsive LNCaP cells, which are more similar to early stage carcinoma, and androgen-insensitive TSU-Pr1 and PC-3 cells, a model for more advanced prostate carcinoma.

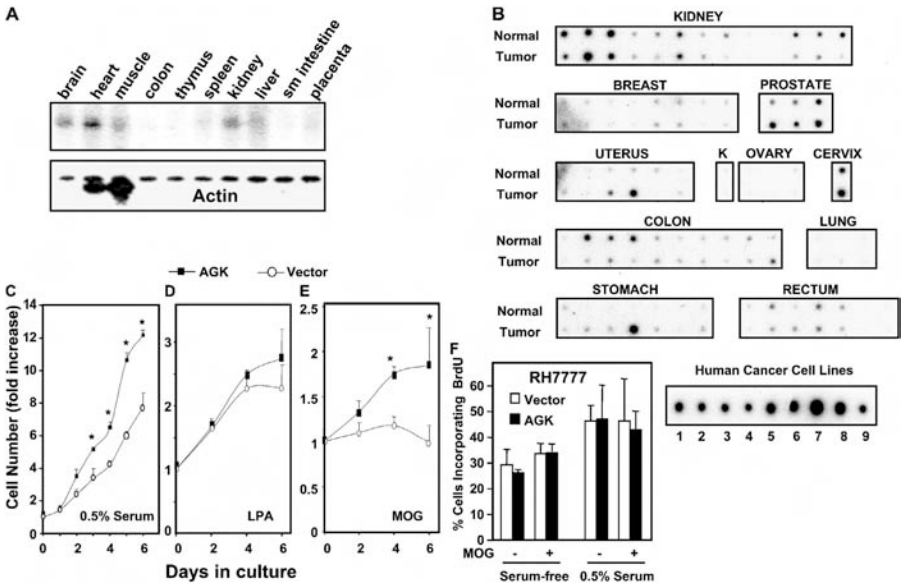


Figure 4. **Expression of hAGK.** (A) Northern blot analysis of hAGK expression in human tissues. Random labeled probe was hybridized to poly(A)$^+$ RNA blots from the indicated human tissues. β-Actin expression was used to confirm equal loading. (B) Matched tumor/normal array analysis of hAGK expression. An array containing cDNA samples from multiple tissues and tumor types as well as nine cancer cell lines was probed with ^{32}P-labeled AGK probe. Each pair of tumor and normal samples came from the same patient. Human cancer cell lines: (1) HeLa; (2) Burkitt's lymphoma, Daudi; (3) chronic myelogenous leukemia; (4) promyelocytic leukemia HL-60; (5) melanoma; (6) lung carcinoma; (7) lymphoblastic leukemia, MOLT-4; (8) colorectal adenocarcinoma, SW480; (9) Burkitt's lymphoma, Raji. There was no specific hybridization to the control nucleic acids, which included ubiquitin cDNA, yeast total RNA, yeast tRNA, *Escherichia coli* DNA, poly(A), human C_ot-1 DNA, and human genomic DNA. (C–E) AGK stimulates proliferation. PC-3 cells stably transfected with vector (open symbols) or AGK (closed symbols) were cultured in serum-free medium supplemented with 0.5% serum (C), 10 μM LPA (D), or 10 μM MOG (E), and cell numbers determined at the indicated days. Data are expressed as fold increase relative to day 0 and are means ± SD. Similar results were obtained in two additional experiments. Asterisks denote significant differences ($P < 0.05$, *t* test). (F) AGK does not enhance proliferation of RH*7777* cells. RH*7777* cells were cotransfected with vector (open bars) or AGK (closed bars) together with GFP at a ratio of 4:1. After 24 h, cells were cultured in serum-free medium or in the presence of 0.5% serum. BrdU was added 16 h later for an additional 3 h. Double immunofluorescence was used to visualize transfected cells and BrdU incorporation into nascent DNA. The proportion of cells incorporating BrdU among total GFP transfected cells was determined. Data are means ± SD of triplicate cultures from a representative experiment. At least three different fields with a minimum of 100 cells/field were scored.

AGK expression enhances cell growth through LPA receptors

Growth promotion is one of the most prominent effects mediated by LPA (Mills and Moolenaar, 2003). Consistent with its ability to increase LPA synthesis, transient or stable expression of AGK enhanced proliferation of diverse cell types, including PC-3 cells (Fig. 4, C–E) and NIH 3T3 fibroblasts (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1). The growth-promoting effect of AGK was observed even in the presence of suboptimal serum concentrations (Fig. 4 C and Fig. S3 A). Although AGK stimulates growth, it had no cytoprotective effects on apoptosis induced by serum deprivation or the anti-cancer drug doxorubicin (Fig. S3 D). As expected, exogenous LPA increased proliferation of both AGK and vector transfectants to the same extent (Fig. 4 D). Addition of MOG to cells cultured in serum-free medium had a minimal effect on vector transfectants and significantly stimulated proliferation of AGK-expressing PC-3 cells (Fig. 4 E). This result is probably due to rapid metabolism and degradation of MOG, and thus only in AGK-expressing cells is sufficient LPA produced and secreted (Fig. 3, F and G) to stimulate proliferation.

FACS analysis revealed that 83 ± 0.4% of the vector transfectants were in Go/G1 phase and 7.0 ± 0.04 and 10 ± 0.4% were in S and G2/M phases, respectively. Overexpression of AGK reduced the fraction of cells in Go/G1 to 73 ± 0.1% and increased the proportion in the S phase by greater than twofold (15 ± 0.1%), without significantly affecting the proportion in the G2/M phase (12 ± 0.1%).

Although it is well established that the mitogenic effects of LPA in many cell types are mediated by binding to its specific GPCRs (Mills and Moolenaar, 2003), intracellular actions have also been suggested (Hooks et al., 2001), possibly as an agonist of the nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPARγ; Zhang et al., 2004). To examine the potential involvement of intracellular actions of LPA generated by expression of AGK, its mitogenic effects were examined in rat hepatoma RH7777 cells that do not express LPA_{1-4} and do not respond to LPA (Fukushima et al., 1998). However, in contrast to PC-3 (Fig. 4, C–E) and NIH 3T3 (Fig. S3 A) cells, RH7777 cells did not show an increase in DNA synthesis in response to expression of AGK as measured by incorporation of BrdU into nascent DNA (Fig. 4 F). Although RH7777 cells express AGK, it is possible that they do not produce sufficient amounts of LPA if MOG is limiting. However, even addition of MOG did not enhance BrdU incorporation in RH7777 cells expressing AGK (Fig. 4 F).

Similar to a previous paper (Lea et al., 2004), GW9662, a selective antagonist of PPARγ, inhibited proliferation of PC-3 cells (Fig. 5 A). However, importantly, it did not abrogate the mitogenic effect of AGK (Fig. 5 A). Consistent with previous studies (Kue et al., 2002), we found that LPA_1, LPA_2, and LPA_3 are expressed in PC-3 cells (unpublished data). It is known that LPA_1 couples to pertussis toxin (PTX)–sensitive Gi, whereas LPA_2 and LPA_3 couple also to Gq. In PC-3 cells, LPA-regulated mitogenic signaling is mediated by Gβγ subunits derived from PTX-sensitive Gi proteins (Bookout et al., 2003). In agreement, PTX pretreatment not only inhibited growth of vector transfectants but it also markedly decreased the growth-promoting effects of AGK (Fig. 5 A). However, AGK expression enhanced cell proliferation even in the presence of PTX.

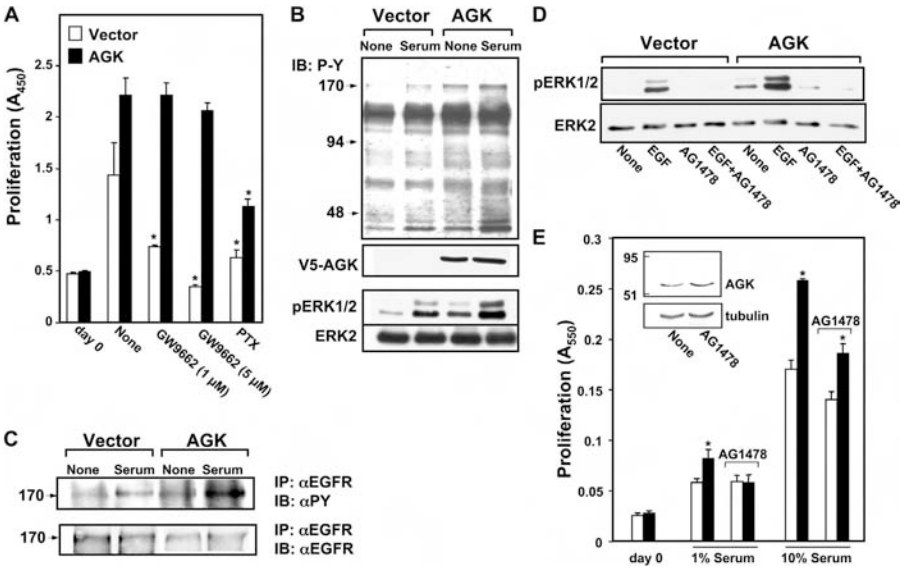


Figure 5. **Regulation of cell growth and EGFR signaling by AGK.** (A) Effect of PTX and the PPARγ antagonist GW9662 on AGK-induced proliferation. PC-3 cells stably transfected with vector (open bars) or AGK (closed bars) were cultured in medium supplemented with 1% serum without or with GW9662 (1 μM or 5 μM) or with PTX (100 ng/ml), and cell proliferation was determined after 6 d with WST-1. Asterisks denote significant differences compared with untreated controls ($P < 0.05$, *t* test). (B) Enforced expression of AGK enhances EGFR tyrosine phosphorylation and stimulates ERK1/2. Serum-starved PC-3 cells stably transfected with vector or AGK were stimulated without or with 10% serum for 10 min, lysed and immunoblotted with anti-phosphotyrosine, anti-V5 antibody, or phospho-specific anti-ERK1/2 antibodies. Blots were stripped and reprobed with ERK2 antibody to demonstrate equal loading. (C) AGK expression induces EGFR transactivation. Lysates from cells treated as in B were immunoprecipitated with anti-EGFR antibody and the immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine or anti-EGFR antibody. (D and E) Blockage of EGFR signaling suppresses ERK activation and cell growth advantage mediated by AGK. (D) Serum-starved PC-3 cells stably transfected with vector or AGK were preincubated for 60 min in the absence or presence of 200 nM AG1478, and then treated with EGF for 10 min. Cell lysate proteins were analyzed by immunoblotting with phospho-specific ERK1/2 antibody. Blots were stripped and reprobed with ERK2 antibody to demonstrate equal loading. (E) PC-3 cells stably transfected with vector or AGK were cultured in medium supplemented with 1 or 10% serum with or without 200 nM AG1478, and cell proliferation was determined after 6 d with crystal violet. Similar results were obtained in two additional experiments. Asterisks denote significant differences ($P < 0.05$, *t* test). (inset) PC-3 cells stably transfected with V5-AGK were incubated for 6 d without (None) or with AG1478, and AGK expression was determined by immunoblotting with anti-V5 antibody. The blot was stripped and reprobed with anti-tubulin as a loading control.

AGK promotes transactivation of EGFR

Many studies have led to the notion that LPA is important in the pathophysiology of prostate carcinoma functioning in an emerging paradigm of cross talk between LPA receptors and the tyrosine kinase EGFR (Prenzel et al., 1999; Mills and Moolenaar, 2003). Therefore, it was of importance to determine whether overexpression of AGK and increased LPA levels resulted in such receptor transactivation leading to enhanced growth.

In serum-starved cells, AGK expression increased tyrosine phosphorylation of several proteins, notably a 170-kD band, which was similarly increased by serum in vector transfectants (Fig. 5 B). Kinetic analysis revealed that the 170-kD tyrosine phosphorylation induced by serum was a rapid event in AGK-expressing cells, clearly evident within 5 min and remaining elevated for at least 60 min (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1). The enhanced tyrosine phosphorylation of the 170-kD protein represented activation of the EGFR, as immunoblotting of anti-EGFR immunoprecipitates with anti-phosphotyrosine revealed increased EGFR tyrosine phosphorylation in cells overexpressing AGK, even in the absence of serum (Fig. 5 C).

AGK-induced extracellular signal related kinase (ERK) 1/2 activation requires EGFR

Previously, it has been suggested that EGFR activation is required for signal relay from LPA receptors to ERK1/2 activation in prostate cancer cells (Prenzel et al., 1999; Kue et al., 2002; Raj et al., 2002). AGK expression markedly increased activation of ERK1/2, as determined with a phospho-specific antibody, which was further enhanced by serum (Fig. 5 B) and EGF (Fig. 5 D and Fig. S3 C). To further confirm that activation of the EGFR was necessary for AGK-stimulated ERK activation, we used the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478. As expected, AG1478 abolished EGFR-induced tyrosine phosphorylation (Fig. S4 B). AG1478 blocked AGK-mediated ERK1/2 phosphorylation (Fig. 5 D) and decreased its mitogenic effect (Fig. 5 E) and also inhibited MOG-stimulated proliferation by 35 ± 4%. Nonetheless, prolonged treatment with AG1478 did not affect AGK protein levels (Fig. 5 E, inset).

Involvement of AGK in motility

Transactivation of the EGFR has also been implicated in motility of cancer cells (Gschwind et al., 2001). In agreement, AGK overexpression enhanced migration of PC-3 cells toward EGF, which was blocked by the EGFR inhibitor AG1478 (Fig. 6 A). AGK also enhanced migration of NIH 3T3 fibroblasts toward serum (Fig. S3 B).

In the Boyden chamber cell migration assay, differences in cell shape and size may affect passage through the pores in the membrane but do not affect the in vitro wound closure assay. AGK expression also enhanced closure of the wounded area, especially in the presence of EGF and the AGK substrate MOG (Fig. 6, B and C). In contrast, wound closure induced by LPA was not affected by AGK expression. AGK-induced wound closure was also blocked by AG1478, supporting a role for EGFR transactivation in AGK-induced migratory responses.

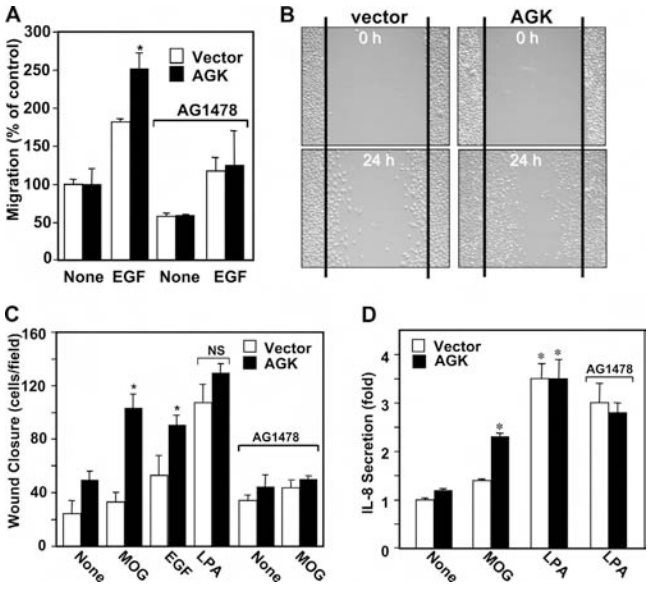


Figure 6. **EGFR is required for AGK-stimulated cell migration toward EGF and wound closure.** (A) PC-3 cells transfected with vector (open bars) or AGK (closed bars) were pretreated without or with 200 nM AG1478 for 20 min and allowed to migrate for 3 h toward EGF (10 ng/ml). The data are means ± SD of two determinations. Similar results were obtained in two independent experiments. (B and C) Monolayers of vector (open bars) or AGK (closed bars) PC-3 transfectants were wounded and treated with vehicle, MOG (10 μM), LPA (10 μM), or EGF (10 ng/ml). Where indicated, cells were also treated with 200 nM AG1478. (B) Representative images of a wound healing assay with vector and AGK-transfected PC-3 cells before and 24 h after treatment with MOG. (C) Migration of cells into the wound was determined after 24 h by processing digital photographs with ImagePro Plus. (D) AGK induces IL-8 secretion. PC-3 cells transfected with vector (open bars) or AGK (closed bars) were serum starved for 24 h and treated in serum-free DME with or without MOG (10 μM) or LPA (1 μM) for 16 h, and IL-8 secretion was measured by ELISA. Where indicated, cells were also treated with 200 nM AG1478. *, $P < 0.05$ by *t* test.

AGK up-regulates IL-8

Expression of the multifunctional cytokine IL-8 correlates with angiogenesis, tumorigenicity, and metastasis of human prostate cancer cells implanted in nude mice (Kim et al., 2001). Similarly, LPA markedly enhanced IL-8 secretion from PC-3 cells. Expression of AGK slightly increased IL-8 release, which was further significantly increased by addition of MOG, the precursor of LPA (Fig. 6 D). The EGFR inhibitor AG1478 only slightly decreased LPA-induced IL-8 secretion, suggesting that this response is independent of EGFR transactivation.

Involvement of endogenous AGK in ERK1/2 activation and cell cycle progression

Serum and EGF induced significant increases in AGK expression as determined by quantitative real-time PCR (Fig. 7 A). It has previously been shown that LPA itself is sufficient to increase its own production in PC-3 cells, indicating the pres-

ence of an autocrine network (Qi et al., 1998). Consistent with an autocrine function for LPA, we found that LPA also increased expression of AGK by threefold in naïve PC-3 cells (Fig. 7 A). To examine the physiological function of AGK, its expression was down-regulated with small interfering RNA (siRNA). siAGK, but not control siRNA, markedly reduced AGK mRNA in PC-3 cells, as determined by QPCR, without influencing expression of SphK1 (Fig. 7 B). Consistent with its role in synthesis of LPA and PA, the most striking effect of down-regulating AGK was reduction of mitochondrial PA and LPA by ~30% (Fig. 7 C). Remarkably, siAGK completely blocked stimulation of ERK1/2 induced by EGF (Fig. 7 D). To rule out off-target effects, we used two additional unrelated siRNAs targeted to different sequences of AGK. $siAGK_2$ and $siAGK_3$ markedly and specifically reduced expression of AGK determined by QPCR (0.2 and 0.16 relative to siControl) without reducing expression of SphK1 (1.1 and 1.0 relative to siControl) or SphK2 (1.1 and 1.0 relative to siControl). Importantly, both of these siRNAs also markedly reduced EGF-induced ERK1/2 activation but did not decrease LPA-induced ERK activation (Fig. 7 E), suggesting that LPA can bypass the effects of down-regulation of AGK. In addition, down-regulation of AGK reduced EGF-stimulated tyrosine phosphorylation of the EGFR (Fig. S3 C).

Down-regulation of AGK reduced EGF-induced wound closure but had no effect on wound closure induced by LPA (Fig. 7 F). siAGK also reduced migration toward EGF but not toward serum (Fig. 7 G). siAGK but not siControl inhibited basal secretion of IL-8 in untreated PC-3 cells and also blocked the small effect of MOG (1.28- and 1-fold stimulation in siControl and siAGK, respectively; Fig. 7 H). However, its effects on EGF or LPA-induced IL-8 secretion were smaller (fold stimulation with EGF is 2.16 and 2.06 and with LPA is 5 and 7.5 in siControl and siAGK, respectively). Similarly, $siAGK_2$ also reduced basal IL-8 secretion without affecting LPA-induced secretion (Fig. 7 H).

Next, we examined the role of endogenous AGK in cell growth regulation. The levels of LPA in serum range from 1 to 6 μM (Baker et al., 2001), and in 10% serum, the level is well below the concentration needed for its mitogenic effects. In agreement with others (Qi et al., 1998), we have found that serum is a more potent mitogen for PC-3 cells than 10 μM LPA (unpublished data). Remarkably, siAGK markedly decreased DNA synthesis as measured by incorporation of BrdU into nascent DNA, whereas nonspecific siRNA had no effect (Fig. 8, A and B). In agreement, cell cycle analysis revealed that after one day in serum-free medium, >75% of PC-3 cells transfected with nonspecific siRNA were in G_o/G_1 phase and only a small fraction were in S and G_2/M phases, which was similar to untreated cells (Fig. 8 C and not depicted). Transfection with siAGK increased the fraction of cells in G_o/G_1 and decreased the proportion in the S phase and the G_2/M phase. In the presence of 10% serum, which markedly increased the proportion of cells in the S phase and G_2/M phase, siAGK, but not control siRNA, further reduced cells in S phase, albeit to a lesser extent than in the absence of serum (Fig. 8 C).

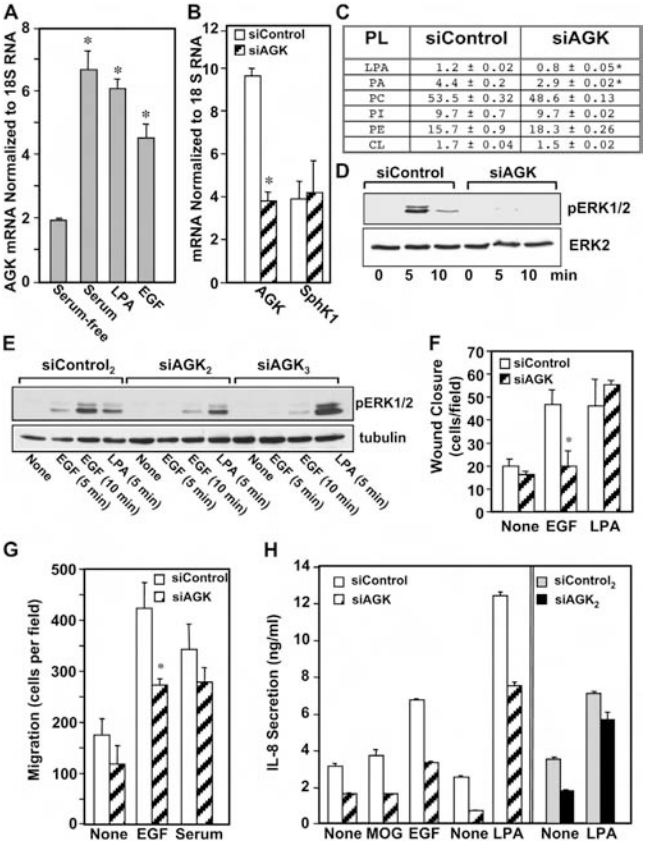
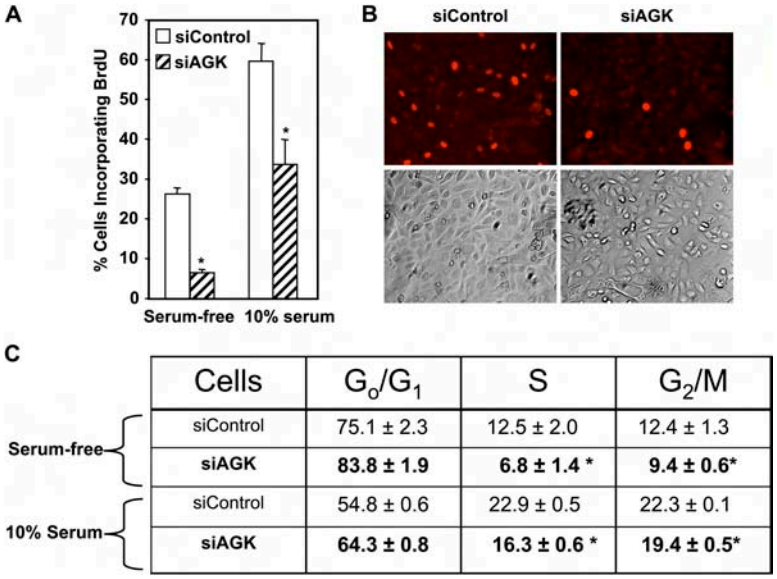


Figure 7. **Effectiveness and specificity of siAGK.** (A) Expression of endogenous AGK. Naïve PC-3 cells were serum starved for 24 h and treated in DME with or without 10% serum, LPA (10 μM), or EGF (100 ng/ml) for 16 h, and AGK mRNA was determined by quantitative real-time PCR. Data were normalized to expression of 18S RNA and are means ± SD of triplicate determinations. *, $P < 0.05$ by *t* test. (B) PC-3 cells were transfected with control siRNA (open bars) or siRNA specific for AGK (hatched bars) and mRNA levels of AGK and SphK1, and 18S RNA was determined by QPCR. (C) Duplicate cultures were labeled with ^{32}Pi for 12 h. Phospholipids were extracted from mitochondria isolated by differential centrifugation. Equal amounts of ^{32}P-phospholipids were separated by TLC, and the indicated phospholipids were quantified with a phosphoimager. The data are expressed as a percentage of total ^{32}P-labeled phospholipids and are means ± SD of duplicate determinations. *, $P < 0.05$ by *t* test. (D) Down-regulation of AGK with siRNA blocks EGF-induced ERK1/2. PC-3 cells were transfected with control siRNA or siRNA specific for AGK and treated without (None) or with 10 ng/ml EGF for the indicated times. Equal amounts of lysate proteins were separated by SDS-PAGE, and ERK1/2 activation was determined by immunoblotting with anti-pERK1/2. Blots were stripped and reprobed with anti-ERK2 as a loading control. (E) PC-3 cells were transfected with $siControl_2$, $siAGK_2$, or $siAGK_3$ (Dharmacon), as described in Materials and methods, and treated with EGF (10 ng/ml) or LPA (10 μM) for the indicated times. Equal amounts of lysate proteins were separated by SDS-PAGE and ERK1/2 activation determined by immunoblotting with anti-pERK1/2. Blots were stripped and reprobed with anti-tubulin as a loading control. Down-regulation of AGK decreases EGF-induced wound closure and migration. (F) Monolayers of PC-3 cells transfected with control siRNA (open bars) or siRNA specific for AGK (hatched bars) were wounded and treated with vehicle (None), EGF (10 ng/ml), or LPA (10 μM), and migration of cells into the wound was determined after 24 h. (G) Cells from duplicate cultures were allowed to migrate for 3 h in Boyden chambers toward vehicle (None), EGF (10 ng/ml), or serum (10%). The data are means ± SD of two determinations. (H) Down-regulation of AGK with siRNA decreases IL-8 secretion. PC-3 cells were transfected with the indicated control siRNAs (open bars and gray bars) or siRNAs specific for AGK (hatched bars and black bars), incubated in serum-free DME without (None) or with MOG (10 μM), LPA (1 μM), or EGF (10 ng/ml) for 16 h, and IL-8 secretion was measured. *, $P < 0.05$ by *t* test.



	Cells	G_0/G_1	S	G_2/M
Serum-free	siControl	75.1 ± 2.3	12.5 ± 2.0	12.4 ± 1.3
	siAGK	**83.8 ± 1.9**	**6.8 ± 1.4 ***	**9.4 ± 0.6***
10% Serum	siControl	54.8 ± 0.6	22.9 ± 0.5	22.3 ± 0.1
	siAGK	**64.3 ± 0.8**	**16.3 ± 0.6 ***	**19.4 ± 0.5***

Figure 8. **Involvement of endogenous AGK in cell proliferation.** (A) PC-3 cells were transfected with control siRNA (open bars) or siAGK (hatched bars) and serum starved for 8 h. After culturing for an additional 16 h in serum-free medium or in medium supplemented with 10% serum, BrdU was added for 3 h and the fraction of cells incorporating BrdU was determined. Data are means ± SD of duplicate cultures from a representative experiment. At least three different fields were scored with a minimum of 100 cells per field. Similar results were obtained in two independent experiments. (B) Representative fluorescent and phase images of siControl and siAGK-transfected cells. (C) Cell cycle analysis. PC-3 cells transfected with control siRNA or siAGK were cultured in serum-free medium or in medium supplemented with 10% serum. After 24 h, cellular DNA was stained with propidium iodide and cell cycle analysis was performed with an Epics XL-MCL flow cytofluorometer (Beckman Coulter). Asterisks indicate significant differences from vector-transfected values as determined by *t* test ($P \leq 0.05$).

Discussion

Several lines of evidence suggest that AGK is a bona fide acylglycerol kinase. First, recombinant AGK catalyzes the phosphorylation of both MOG and DAG in vitro, producing LPA and PA, respectively. In agreement, bacterially expressed, purified recombinant AGK phosphorylated DAG as well as MOG (Waggoner et al., 2004). Second, overexpression of AGK increases cellular levels of LPA and PA. Third, and conversely, down-regulation of AGK reduces their intracellular levels. Fourth, overexpression of kinase dead AGK, although localized similarly to the mitochondria as wild-type AGK, does not increase acylglycerol kinase activity nor produce LPA.

Prostate carcinomas often possess an autocrine stimulatory loop in which the transformed cells express high levels of EGFR and also produce activating ligands. One such ligand might be the bioactive phospholipid LPA, which stimulates prostate cancer cell proliferation, migration, and survival, not only by acting on its cognate GPCRs but also by stimulating metalloproteinase activity and proteolytic EGF precursor processing leading to EGFR transactivation (Gschwind et al., 2001). Our identification of AGK as a lipid kinase that produces LPA and PA and is highly expressed in prostate tumors may have important clinical implications with regard to advanced prostate cancer. AGK not only regulates mitogenic EGFR signaling that plays important roles in androgen-refractory metastatic prostate cancer but also stimulates cell motility and EGFR-independent secretion of the pluripotent cytokine IL-8. Indeed, blockade of the EGFR in PC-3 cells inhibited tumor growth and invasion and also down-regulated expression of IL-8 within the tumors (Karashima et al., 2002).

Interestingly, the endocannabinoids anandamide and 2-AG potently inhibit proliferation and cause apoptosis of PC-3 and DU145 prostate cancer cells (Melck et al., 2000). Because AGK can phosphorylate 2-AG converting it to LPA, it may regulate the dynamic levels of these counterregulatory lipids that have been shown to play opposing roles in growth and survival of prostate cancers. Of note, LPA_3 was originally cloned from prostate cancer cells (Im et al., 2000), which is concordant with the ability of LPA to induce autocrine proliferation of these cells (Xie et al., 2002). Moreover, PC-3 cells express $LPA_{1\text{-}3}$ receptors, thus providing AGK with the potential to activate numerous downstream growth signaling pathways.

The mitogenic responses of some mammalian cells to LPA may be $LPA_{1\text{-}4}$ independent (Hooks et al., 2001). However, in prostate cancer cells, LPA transduces mitogenic signals via activation of $G_{\alpha i}$ and $G\beta\gamma$ subunits (Kue and Daaka, 2000; Bookout et al., 2003). Importantly, the expression of GRK2ct inhibited PC-3 tumor formation in animals (Bookout et al., 2003). Several lines of evidence indicate that the growth-promoting effects of AGK are mediated via LPA receptors. In RH7777 cells, which are devoid of $LPA_{1\text{-}4}$, expression of AGK had no effect on DNA synthesis, even in the presence of exogenous MOG. Moreover, PTX pretreatment decreased the growth-promoting effects of AGK. Our results are similar to others showing that PTX not only inhibits proliferation of PC-3 cells induced by LPA but also inhibits the effect of serum more drastically (Kue and Daaka, 2000). Furthermore, consistent with a previous study (Lea et al., 2004), GW9662, a selective antagonist of PPARγ, inhibited proliferation of vector-transfected PC-3 cells, and yet it did not abrogate the mitogenic effect of AGK. Our results are consistent with the notion that production and secretion of LPA by AGK induces Gi-dependent proliferation, likely through LPA receptors. Remarkably, down-regulation of AGK drastically reduced ERK1/2 activation induced by EGF, which, as expected, was bypassed by addition of the AGK product, LPA. Our data suggest that AGK plays an important role in EGF-induced mitogenic ERK signaling. AGK also phosphorylates DAG to produce the bioactive mediator PA, which regulates numerous biological processes including Raf translocation to the plasma membrane (Rizzo et al., 1999) and activation of mTOR (Fang et al., 2001). Our results also imply that specific pools of PA may play important roles in growth signaling.

Although the physiological function of LPA and PA generation in the mitochondria is not clear, a LPA phosphatase with 28.5% amino acid identity to human prostatic acid phosphatase is also localized to the mitochondria (Hiroyama and Takenawa, 1999). This LPA phosphatase has been suggested to regulate lipid metabolism in mitochondria by hydrolysis of LPA to monoacylglycerol (Hiroyama and Takenawa, 1999). Previous studies suggest that mitochondria-produced LPA can leave this organelle and be transported to the ER in the presence of liver fatty acid binding protein, can be secreted, and/or can be converted to PA (Haldar and Lipfert, 1990; Chakraborty et al., 1999; Hiroyama and Takenawa, 1999). In addition, LPA generated by prostate cancer cells in response to mitogenic stimuli can be secreted (Qi et al., 1998; Gibbs et al., 2000; Kue and Daaka, 2000; Kue et al., 2002; Xie et al., 2002).

It has been demonstrated that in both PC-3 and DU145 prostate cancer cells, agonists induce 18:1 LPA formation that is then released into the medium (Xie et al., 2002). As Du145 and PC-3 cells express $LPA_{1\text{-}3}$, it was suggested that 18:1 LPA can act as an autocrine mediator (Xie et al., 2002), yet the critical enzymes involved have not been identified. Our data suggest that AGK could be a missing link. Production of LPA by AGK, which in turn transactivates the EGFR, can amplify mitogenic and survival signals. Moreover, expression of AGK is stimulated by EGF, serum, and even by LPA itself, thereby providing a positive feed-forward stimulus that could further enhance EGFR-dependent and -independent processes important for cancer progression. Therefore, targeting AGK, which is upstream of the EGFR, could offer additional therapeutic benefits in treatment of androgen-independent prostate cancer.

Materials and methods

Cloning of an AGK

An EST (AW321722) was identified that contained an open reading frame with 25% identity and 50% similarity to hSphK2 from aa 133 to 256. 5′ and 3′ RACE were performed using the GeneRacer kit (Life Technologies) to obtain the sequence of the full-length open reading frame. A cDNA with a complete open reading frame was cloned from a human kidney cDNA library encoding a 422–amino acid polypeptide with a calculated molecular mass of 46,400 D (Fig. S1). A nearly identical mouse homologue (CAC06108) was also identified.

The predicted sequence of this human protein and its mouse homologue identified from the mouse database (CAC06108) are 95% identical and both show sequence similarity to SphKs, especially in the five conserved SphK domains (Liu et al., 2000, 2002). We previously noted that conserved regions 1–3 of SphKs have high sequence homology with the catalytic domain of DAGKs (DAGKc; Liu et al., 2002). This region (aa 65 to 191 of the new kinase) contains the $GDGXXXEXXXGXXXRX_nK$ ($n = 7$, 8) motif, present in the catalytic domain of SphKs (SphKc; Liu et al., 2002), which is reminiscent but distinct from the sequence GGDGXXG previously suggested to be part of the ATP binding site of DAGKc (Topham and Prescott, 1999). Of note, a lysine residue downstream of the glycine-rich region, which is conserved in the ATP binding sites of protein kinases (Hanks et al., 1988) and absent in DAGKc, is also present in SphKc and in this new lipid kinase (Fig. S1). However, Clustal W alignment revealed that hSphK1 and hSphK2 are more closely related to each other than to this new putative lipid kinase. Pairwise comparisons of the conserved subdomains of SphK1/SphK2, new lipid kinase/SphK1, new lipid kinase/SphK2, new lipid kinase/CERK, and new lipid kinase/DAGK indicated sequence identities of 53, 29, 23, 26, and 24%, respectively. These comparisons suggest that this new lipid kinase may be unique. A search of the human genome database revealed that the gene encoding this lipid kinase is located on chromosome 7q34, whereas SphK1 and SphK2 have been localized to chromosomes 17q25.2 and 19q13.2, respectively.

Cell culture and transfection

Human PC-3 prostate cancer cells (CRL-1435; American Type Culture Collection), NIH 3T3 fibroblasts (CRL-1658; American Type Culture Collection), rat hepatoma RH*7777* cells (provided by X. Fang, Virginia Commonwealth University, Richmond, VA), and human embryonic kidney cells (HEK 293; CRL-1573; American Type Culture Collection) were seeded at $4–5 \times 10^5$ cells per well in 6-well plates and transfected with Lipofectamine PLUS for NIH 3T3, HEK 293, and RH*7777* cells and Lipofectamine 2000 for PC-3 cells, according to the manufacturer's instructions (Life Technologies).

Catalytically inactive AGK

The QuikChange site-directed mutagenesis kit (Stratagene) was used to prepare catalytically inactive AGK (G126E) by mutating the conserved glycine in the glycine-rich loop of the ATP binding site (forward primer, 5′-TTGGAGGAGGAGATGAGACACTGCAGGAGGTT-3′, and reverse primer, 5′-AACCTCCTGCAGTGTCTCATCTCCTCCTGCAA-3′). The mutation was confirmed by sequencing.

siRNA transfection

AGK expression was down-regulated with sequence-specific siRNA. siRNA target sequence for AGK (siAGK: 5′-AACAGATGAGGCTACCTTCAG-3′) and control siRNA (5′-TTCTCCGAACGTGTCACGT-3′) were obtained from QIAGEN. In some experiments, cells were transfected with two additional AGK siRNAs ($siAGK_2$: 5′-GAGGCTACCTTCAGTAAGA-3′; $siAGK_3$: 5′-GGAGAGACCAGTAGTTTGA-3′) and siControl (non-targeting siRNA with at least four mismatches to all human and mouse genes from Dharmacon). Cells (3×10^5) were transfected in 6-well dishes for 3–4 h with the RNA duplexes (200 nM) using Oligofectamine (Life Technologies) according to the manufacturer's protocol. $90 \pm 2\%$ of the cells were transfected as determined with siGLO RISC-Free siRNA (Dharmacon).

Real-time PCR

Quantitative real-time PCR was performed on a real-time PCR machine (model Taqman ABI 7900; Applied Biosystems) with the following primers/probes: AGK forward primer, 5′-CGAAGGCTTGCGTCCTACTG-3′; reverse primer, 5′-TGGTGGACAGCTGCACATCT-3′; probe, 5′-CACAACCACAGGATGCCCTTTCCC-3′ (Integrated DNA Technologies); premixed primer-probe set for hSphK1 was purchased from Applied Biosystems. Ribosomal RNA (18S rRNA) measured using TaqMan assay reagents served as endogenous control.

Lipid kinase activity

Lipids (100 nmol) were dried under N_2 and resuspended in 180 μl of buffer containing 100 mM MOPS, pH 7.2, 2 mM EGTA, 15 mM NaF, 2 mM orthovanadate, 50 mM NaCl, 250 mM sucrose, 0.03% deoxycholate, and 1:500 diluted protease inhibitor cocktail (Sigma-Aldrich). After brief sonication, 10 μl lysates (10 μg) and 10 μl γ-[^{32}P]ATP (10 μCi, 1 mM) containing $MgCl_2$ (10 mM) were added and reactions were performed for 30 min at 37°C. ^{32}P-labeled lipids produced were extracted into 0.8 ml $CHCl_3$/MeOH/concentrated HCl (100:200:1, vol/vol), and phase separation was effected by adding 0.25 ml 2 M KCl and 0.25 ml $CHCl_3$. Aliquots of the organic phases were analyzed by TLC on silica gel G60 with $CHCl_3$/acetone/methanol/acetic acid/water (10:4:3:2:1, vol/vol) as solvent and the radioactive spots corresponding to migration of standards were quantified with an FX Molecular Imager (Bio-Rad Laboratories). In some experiments, SphK1 (Liu et al., 2000), SphK2 (Liu et al., 2000), ceramide, and DAGKs (Sugiura et al., 2002) were measured exactly as described.

AGK activity in immunoprecipitates

HEK 293 cells were seeded in 10-cm dishes and transiently transfected with vector or V5-tagged AGK. 24 h later, cells were lysed by sonication in buffer containing 100 mM MOPS, pH 7.2, 2 mM EGTA, 2 mM orthovanadate, 2 mM β-glycerophosphate, 150 mM NaCl, 250 mM sucrose, and 1:500 diluted protease inhibitor cocktail. Lysates were cleared by centrifugation and 400 μg of protein in 250 μl was incubated with 1 μg anti-V5 (Invitrogen) for 4 h at 4°C. Protein A/G PLUS–Agarose beads (10 μl; Santa Cruz Biotechnology, Inc.) were added and incubated for an additional 1 h. The beads were washed four times with the same buffer, resuspended in 10 μl, and AKG activity was determined.

^{32}P labeling of cellular phospholipids

Vector and kinase PC-3 transfectants were grown to 80–90% confluency in 100-mm dishes, incubated for 2 h with 40 μCi/ml ^{32}Pi in phosphate-free DME at 37°C, and washed and incubated for a further 2 h in 4 ml

phosphate-free DME. Medium was removed and after brief centrifugation lipids were extracted from a 3-ml aliquot of the medium by addition of 10.8 ml chloroform/methanol/concentrated HCl (100:200:1, vol/vol), followed by 3.6 ml each of chloroform and 2 M KCl. Lipids were also extracted from the cells after washing and scraping into 1.2 ml cold methanol/concentrated HCl (100:1), followed by addition of 0.6 ml chloroform. After vigorous vortexing, 0.6 ml $CHCl_3$ and 0.5 ml H_2O was added. Phases were separated by addition of 0.6 ml 2 M KCl. The organic phases were transferred to siliconized glass tubes and the aqueous phases reextracted with 0.6 ml $CHCl_3$. Aliquots containing 50,000 cpm were separated by two-dimensional TLC (Yokoyama et al., 2000). Radioactive spots were identified by comparison to standard phospholipids and quantified with a phosphoimager. In some experiments, phospholipids were separated by one-dimensional TLC using $CHCl_3$/methanol/water/ammonium hydroxide (120:75:6:2, vol/vol; Liu et al., 2003).

Northern analysis and matched tumor/normal expression array
Poly(A)$^+$ RNA blot of multiple adult human tissues (CLONTECH Laboratories, Inc.) was used for Northern blotting analysis of AGK expression. The blot was hybridized with a probe prepared by labeling the PCR product with γ-[^{32}P]dCTP in ExpressHyb buffer (CLONTECH Laboratories, Inc.) at 65°C overnight. A matched tumor/normal expression array (CLONTECH Laboratories, Inc.) was similarly probed with radiolabeled full-length AGK.

Chemotactic motility
Chemotaxis was measured in a modified Boyden chamber using collagen-coated polycarbonate filters (25 × 80 mm, 8-μM pore size) as previously described (Wang et al., 1999).

In vitro wound closure assay
Confluent monolayers of PC-3 cells were serum starved for 24 h, wounded by making a uniform scratch with a pipet tip, and washed to remove detached cells. Wound closure was monitored after 24 h in serum-free medium by determining the number of cells migrating into the wound using ImagePro Plus software to analyze digital images from an inverted phase microscope.

Cell proliferation assays
PC-3 cell proliferation was determined with crystal violet (Olivera et al., 1999). In some experiments, cell growth was measured by adding WST-1 reagent (Roche) and incubating at 37°C for 3 h. Absorbance was measured at 450 nm with background subtraction at 650 nm. BrdU incorporation and analysis of cell cycle profile by flow cytometry were performed exactly as described previously (Olivera et al., 1999).

IL-8 secretion
PC-3 cells were serum starved overnight, and after stimulation in serum-free DME for 16 h, media was collected and briefly centrifuged to remove cells. Secreted IL-8 was determined with the Quantikine IL-8 ELISA kit (R&D Systems).

Immunofluorescence and confocal microscopy
Cells were grown on glass coverslips and transfected with vector or V5-tagged AGK. 24 h later, cells were incubated with 200 nM MitoTracker red CMXRos (Molecular Probes) to stain mitochondria and fixed in 3% PFA in PBS containing 0.1% Triton X-100. ER was visualized with polyclonal rabbit anti-calnexin antibody followed by anti–rabbit IgG-FITC. Transfected cells were visualized simultaneously with anti-V5 antibody (1:500; Invitrogen) followed by a secondary anti–mouse antibody conjugated with FITC or Texas red (Molecular Probes), respectively. Coverslips were mounted on glass slides using an Anti-Fade kit (Molecular Probes) and examined by confocal microscopy. Images were collected with a laser scanning microscope (model IX70; Olympus) equipped with argon (488 nm) and krypton (568 and 647 nm) lasers and a 60×/1.4 NA PlanApo lens. Quantitative image analysis was performed using Metamorph image processing software.

Subcellular fractionation
Cells transfected with vector or AGK were Dounce homogenized in buffer containing 20 mM Hepes, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. Subcellular fractionation was performed by differential centrifugation at 4°C as described previously (Le Stunff et al., 2002). In brief, lysates were centrifuged at 1000 *g* for 5 min to remove unbroken cells and nuclei (P1, nuclei and unbroken cells); postnuclear supernatants were centrifuged at 5,000 *g* for 10 min (P2, mitochondria); followed by 17,000 *g* for 15 min (P3, intracellular membrane fraction containing ER and Golgi). The remaining supernatant was centrifuged at 100,000 *g* for 1 h to obtain plasma membranes. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies as described in the figure legends.

Immunoprecipitation
PC-3 cells were lysed in buffer containing 25 mM Hepes, pH 7.5, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM dithiothreitol, 1 mM PMSF, and 10 μg/ml leupeptin. Lysates were cleared by centrifugation at 10,000 *g* for 10 min and incubated with 2 μg anti-EGFR antibody for 2 h at 4°C. 20 μl of protein A/G–Sepharose beads (Santa Cruz Biotechnology, Inc.) were added and incubated for an additional 1 h at 4°C. Sepharose beads were washed and boiled in SDS sample buffer, and bound proteins were analyzed by Western blotting.

Online supplemental material
Fig. S1 shows a Clustal W alignment of AGK with other lipid kinases. Fig. S2 demonstrates the mitochondrial localization of AGK and catalytically inactive AGK and confirms that the mutant has no kinase activity. Fig. S3 shows the effect of AGK on growth and survival of NIH 3T3 cells. Fig. S4 shows regulation of EGFR phosphorylation by AGK. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1.

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Critical role of acylglycerol kinase in epidermal growth factor-induced mitogenesis of prostate cancer cells

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Abstract

The bioactive phospholipids, LPA (lysophosphatidic acid) and PA (phosphatidic acid), regulate pivotal processes related to the pathogenesis of cancer. Recently, we cloned a novel type of lipid kinase that phosphorylates monoacylglycerols (such as 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand) and diacylglycerols, to form LPA and PA, respectively. This AGK (acylglycerol kinase) is highly expressed in prostate cancer cell lines and the results reviewed here suggest that AGK might be a critical player in the initiation and progression of prostate cancer. Intriguingly, down-regulation of endogenous AGK inhibited EGF (epidermal growth factor), but not LPA-induced ERK1/2 (extracellular-signal-regulated kinase 1/2) activation and progression through the S-phase of the cell cycle. In this review, we will summarize the evidence demonstrating that AGK amplifies EGF growth signalling pathways that play an important role in the pathophysiology of prostate cancer. Because LPA has long been implicated as an autocrine and paracrine growth stimulatory factor for prostate cancer cells, the identification of this novel lipid kinase that regulates its production could provide new and useful targets for preventive or therapeutic measures.

LPA (lysophosphatidic acid): a potent mitogen for prostate cancer

For over 50 years, the primary form of therapy for advanced prostate cancer has targeted the androgen receptor. However, there have been few cures and virtually all patients with metastatic prostate cancer treated with androgen deprivation will progress to androgen independence. However, it is still not fully understood why prostate cancer cells eventually become androgen independent, resistant to therapy, and ultimately cause the death of the patient. The most likely way to develop new and effective therapies for prostate cancer is to improve our understanding of the processes leading to the initiation and progression of this disease. Other factors responsible for the initiation and progression of prostate cancer remain poorly understood, although growth factors, such as EGF (epidermal growth factor) and IGF-II (insulin-like growth factor-II), and hormones have been implicated in the growth and survival of prostate cancer cells [1].

A recent addition to the ranks of prostate mitogenic factors is LPA, a major mitogen in serum. Indeed, the ability of serum to stimulate cell growth appears to be mediated to a large extent by LPA [2–4], and the related lysophospholipid, S1P (sphingosine 1-phosphate), which has a similar structure to LPA but with a sphingosine rather than glycerol backbone, and whose importance in cell growth and survival was discovered in our laboratory [5]. Both LPA and S1P regulate an array of cellular processes related to pathogenesis of cancer, especially prostate and ovarian cancers, including initiation and regulation of proliferation, enhancement of survival, suppression of apoptosis, cytoskeleton reorganization and tumour cell motility and invasiveness [2,3,5]. LPA is a potent mitogen for PC-3 prostate cancer cells, which are androgen independent and models for more advanced carcinomas [6]. Growth and signalling patterns in these cells in response to serum resemble those mediated by LPA and are dependent on G_i $\beta\gamma$ subunits, suggesting an important regulatory role for LPA in the growth of prostate cancer cells [7]. It has also been suggested that LPA responsiveness might be enhanced in more advanced carcinoma [8].

LPA as a ligand for a new class of GPCRs (G-protein-coupled receptors)

Progress in understanding LPA actions was accelerated by the discovery that it is a ligand of several GPCRs [9]. To date, there are three established LPA receptors, LPA_1, LPA_2 and LPA_3 [4,9,10], which are coupled to a variety of G-proteins and regulate diverse cellular responses [4,10]. Expression of LPA receptors correlates with more advanced prostate cancer cell lines [8] and LPA_2 and LPA_3 are aberrantly expressed in ovarian cancer cells [11,12], indicating their potential role in the pathophysiology of cancer. Recently, a fourth putative LPA receptor was described (LPA_4/GPR23/P2Y9) which is distinct from the other LPA receptors [13]. LPA also has

Key words: acylglycerol kinase, epidermal growth factor (EGF), G-protein-coupled receptor (GPCR), lysophosphatidic acid (LPA), phosphatidic acid (PA), prostate cancer.

Abbreviations used: AGK, acylglycerol kinase; EGF, epidermal growth factor; EGFR, EGF receptor; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; GPCR, G-protein-coupled receptor; LPA, lysophosphatidic acid; mTOR, mammalian target of rapamycin; PA, phosphatidic acid; PPAR-γ, peroxisome proliferator-activated receptor-γ; PTX, pertussis toxin; S1P, sphingosine 1-phosphate.

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a novel intracellular function as a high-affinity ligand for PPAR-γ (peroxisome proliferator-activated receptor-γ), a transcription factor that regulates genes controlling energy metabolism [14] and can exacerbate mammary gland tumour development [15].

How does LPA so profoundly influence proliferation of so many types of cells including prostate cancer cells?

LPA can stimulate cell proliferation by several interactive mechanisms: (i) LPA enhances SRE (serum response element) activity in the promoters of immediate early growth-related genes [16]; (ii) LPA stimulates secretion of polypeptide growth factors, such as EGF and IFG-II [17]; (iii) LPA enhances survival and suppresses apoptosis by reducing levels of the apoptosis-promoting protein Bax [18]; (iv) LPA can also sensitize some types of cells to the growth promoting effects of polypeptide growth factors; and (v) finally, LPA can stimulate EGFR (EGF receptor) transactivation by enhancing metalloproteinase activity and processing of proHB-EGF to EGF [19]. Thus, in addition to actions through conventional GPCR signalling pathways, LPA receptors can indirectly regulate cell functions by transactivating the EGF tyrosine kinase receptor [19–21]. This cross-communication between different signalling systems is not only important for the growth promoting activity of LPA [20,21], it may also be a clue to its pathophysiological role in prostate cancer [19], head and neck squamous cell carcinoma [22], and kidney and bladder cancer [23].

LPA and signal transduction

Downstream events linking LPA to growth and survival are complex and to some extent are reminiscent of mitogenic signalling by other GPCRs. Most studies indicate that LPA_1 and LPA_2 can couple to the $G_{i/o}$, $G_{12/13}$ and Gq families, whereas it appears that although LPA_3 is also linked to activation of $G_{i/o}$ and Gq, it does not couple efficiently with $G_{12/13}$. LPA-induced activation of $G\alpha_i$, which is sensitive to PTX (pertussis toxin), leads to decreased cAMP, activation of the ERK (extracellular-signal-regulated kinase) cascade, and potentially tyrosine phosphorylation and proliferation. $G\alpha_i$ as well as $G\alpha_q$ can link to phospholipase C, whereas LPA-induced activation of $G\alpha_{12/13}$, which directly binds to Rho guanine nucleotide exchange factor and activates the small GTPase Rho, leads to cytoskeletal rearrangements and changes in cell migration, invasion and transformation (reviewed in [4]).

Cross-communication between receptor signalling cascades enhances the ability of cells to respond appropriately to different stimuli. In the PC-3 human prostate cancer cell line, LPA stimulates proliferation by activation of ERK1/2 and the tyrosine kinase activity of EGFR. The pathophysiological significance of this mechanism is demonstrated by inhibition of constitutive EGFR activity upon treatment of PC-3 cells with a metalloproteinase inhibitor [19].

Although abundant evidence has been put forward suggesting that the mitogenic effect of LPA is mediated through LPA_1 [17], LPA_2 [11,24] or LPA_3 [25], using degradation-resistant phosphonate analogues of LPA, stereoselective agonists of the LPA receptors, and LPA receptor null cells, Lynch and co-workers demonstrated that the mitogenic effects of LPA are independent of LPA_1–LPA_3 [26]. Thus, similar to many reports showing that the mitogenic effect of S1P might be independent of its receptors [27,28], LPA may also have direct intracellular actions. In this regard, a novel intracellular action of LPA has been uncovered. Some evidence suggests that membrane fission, which occurs whenever a vesicle is produced, is controlled by endophilin I, a cytosolic protein that converts LPA into PA (phosphatidic acid) by the addition of the unsaturated fatty acid, arachidonate [29]. This results in negative membrane curvature by converting an inverted-cone-shaped lipid (LPA) into a cone-shaped lipid (PA) in the cytoplasmic leaflet of the bilayer, mediating synaptic vesicle invagination and fission [29].

LPA metabolism: intracellular production and extracellular release

Because LPA is present at high levels in human ascites fluid [30], it is considered to be a mediator as well as an indicator of ovarian cancer [11,12]. LPA has long been known to be produced in cells as an intermediate in lipid synthesis. While bioactive LPA can also be produced from PA by phospholipases in ovarian and prostate cancer cells [31,32], an important recent discovery relevant to cancer was that autotaxin, a secreted protein known to be involved in tumour invasion, vascularization and metastasis, is a lysophospholipase D that converts extracellular lysophosphatidylcholine into LPA [33]. This finding adds support to the notion that LPA plays an important role in tumorigenesis.

Yet another potential pathway for synthesis of LPA is the phosphorylation of monoacylglycerols by a specific lipid kinase [34], an enzyme that has remained an enigma for more than 40 years. We have now cloned and characterized a new lipid kinase which we called AGK (acylglycerol kinase) since it catalyses the phosphorylation of both monoacylglycerol to form LPA and diacylglycerol to produce PA, another potent lipid second messenger that mediates mitogenic activation of mTOR (mammalian target of rapamycin) signalling [35].

AGK catalyses the phosphorylation of acylglycerols to generate LPA and PA

Because sequence comparisons revealed that AGK is related to the diacylglycerol kinase family and the sphingosine kinase family, it was necessary to establish that it was a *bona fide* AGK. Importantly, when AGK, but not a kinase dead mutant of AGK, was overexpressed, intracellular levels of LPA and PA were increased, without detectable effects on levels of ceramide, sphingosine, or S1P [36]. In agreement, monoacylglycerols and diacylglycerols are substrates for recombinant AGK, while neither sphingosine nor ceramide

were phosphorylated. Furthermore, when AGK expression was down-regulated by transfection with siRNA (small interfering RNA) targeted to AGK, LPA and PA levels were decreased. Interestingly, AGK has a similar substrate preference profile as a previously described crude bovine monoacylglycerol kinase activity [37,38], showing higher activity with acylglycerols containing a C_{18} fatty acid with a single double bond, although arachidonylglycerol, an endogenous cannabinoid containing a C_{24} fatty acid with four double bonds, was a reasonably good substrate.

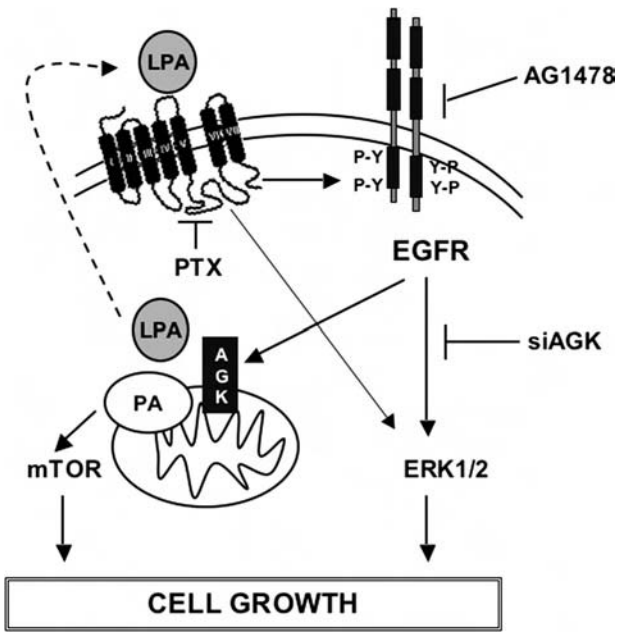
AGK: a key player in survival signals induced by EGF

Proliferation of many types of cancer cells is controlled in part by an autocrine stimulatory loop, since EGFR is often overexpressed in transformed cells that also produce ligands that can transactivate the EGFR by activating their own receptors. Several lines of evidence suggest that AGK, which is highly expressed in prostate cancers, could play a role in prostate cancer progression. Overexpression of AGK in prostate cancer cells increased the formation and secretion of LPA, resulting in transactivation of EGFR and activation of the downstream MAPK (mitogen-activated protein kinase) signalling pathway leading to increased cell growth. Importantly, when endogenous AGK expression was ablated in these prostate cancer cells, EGF-induced ERK1/2 activation and cell proliferation were markedly inhibited [36]. Moreover, down-regulation of AGK decreased EGFR-mediated cell motility, which plays an important role in androgen-refractory prostate cancer.

The endocannabinoids anandamide and 2-arachidonylglycerol are known to induce apoptosis of PC-3 and DU145 prostate cancer cells [39]. As AGK can phosphorylate 2-arachidonylglycerol, converting it into LPA, it can influence the dynamic levels of these counterregulatory lipids that have opposing effects on growth and survival of prostate cancers. Mono-oleoylglycerol, the best substrate for AGK, is phosphorylated to form $C_{18:1}$ LPA, and LPA species with unsaturated fatty acids, in particular, $C_{18:1}$ and $C_{18:2}$ LPA, are much more potent than their saturated counterparts in stimulating the growth of ovarian [40] and prostate cancer cells [32]. In this regard, it has been suggested that increased LPA species with unsaturated fatty acid chains may be associated with late-stage or recurrent ovarian cancer [41]. LPA with unsaturated fatty acids preferentially stimulates LPA_3, whereas LPA_1 and LPA_2 receptors show broader ligand specificities [42]. Prostate cancer cells express LPA_1–LPA_3 receptors and thus AGK can potentially regulate numerous growth signalling pathways downstream of these receptors. Although the mitogenic effects of LPA can be LPA_1–LPA_4 independent [26], in prostate cancer cells, LPA transduces G-protein-dependent mitogenic signals [7,43]. In agreement, our recent results suggest that the growth promoting effects of AGK are mediated via LPA receptors [36]. First, in cells lacking expression of any of the LPA receptors, AGK had no effect on DNA synthesis. Secondly, PTX pretreatment

Figure 1 | Cross-talk between AGK and EGF signalling pathways important for cell growth

siAGK, small interfering RNA targeted to AGK.



decreased the growth promoting effects of AGK. In agreement, PTX inhibits proliferation of PC-3 cells induced by LPA and serum [7]. Thirdly, a selective antagonist of PPAR-γ inhibited proliferation of vector transfected PC-3 cells, yet it did not abrogate the mitogenic effect of AGK.

Down-regulation of AGK reduced ERK1/2 activation induced by EGF, but not by LPA, the AGK product, suggesting that AGK plays an important role in EGF-induced mitogenic ERK signalling. Nonetheless, AGK also phosphorylates diacylglycerol to produce the bioactive mediator PA, which regulates numerous biological processes including Raf translocation to the plasma membrane [44], activation of mTOR [35,45], vesicle transport [46] and cytoskeletal structure [47]. A recent study demonstrated that PA produced at the ER (endoplasmic reticulum) in yeast is not only an essential ubiquitous metabolic intermediate but also an important signalling lipid [48]. PA on the ER is directly bound to the soluble transcriptional repressor Opi1p to maintain it in an inactive state outside the nucleus. Metabolism of PA releases Opi1p and allows its nuclear translocation and repression of target genes that regulate phospholipid biosynthesis. Our results also imply that specific pools of PA may play important roles in signalling in mammalian cells.

At first glance, localization of AGK in the mitochondria seemed odd. However, it should be noted that an LPA phosphatase with 28.5% amino acid identity to human prostatic acid phosphatase is also localized to the mitochondria [49], and regulates lipid metabolism in the mitochondria by degrading LPA to monoacylglycerol [49]. More than a decade ago, it was suggested that LPA produced in the mitochondria can be transported to the ER in the presence of liver fatty acid binding protein, be secreted and/or converted into PA

[49–52]. Moreover, prostate cancer cells can secrete LPA generated by mitogenic stimuli [6–8,32,53,54]. Because these cells also express LPA_1–LPA_3, it has been suggested that LPA can act as an autocrine mediator [32]. However, the enzymes that produce LPA in prostate cancer cells have not been conclusively identified. Our results suggest that production of LPA by AGK, which in turn transactivates the EGFR, can amplify mitogenic and survival signals. In addition, EGF, serum and LPA itself increase the expression of AGK, thus acting in a positive feed-forward manner that could enhance EGFR-dependent and -independent processes important for cancer progression (Figure 1). Hence, targeting AGK could offer additional therapeutic benefits in treatment of androgen-independent prostate cancer.

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Glycosphingolipids and cell death

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Sphingolipids have been implicated in various cellular processes including growth, cell-cell or ligand-receptor interactions, and differentiation. In addition to their importance as reservoirs of metabolites with important signaling properties, sphingolipids also help provide structural order to plasma membrane lipids and proteins within the bilayer. Glycosylated sphingolipids, and sphingomyelin in particular, are involved in the formation of lipid rafts. Although it is well accepted that ceramide, the backbone of all sphingolipids, plays a critical role in apoptosis, less is known about the biological functions of glycosphingolipids. This review summarizes current knowledge of the involvement of glycosphingolipids in cell death and in other pathological processes and diseases.
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Introduction

Glycosphingolipids (GSL) are lipid components of membranes that are important for the proper development of vertebrates. They are involved in multiple processes, including cell type specific adhesion, cell-cell interaction, embryogenesis, and development and differentiation of neuronal cells and leukocytes [1]. GSL can also serve as binding sites for several viruses, bacteria, and bacterial toxins [2]. Different tissues display different GSL patterns on the cell surface which can be dramatically altered during development [1]. A further modulation can be seen during pathological processes such as tumor development. GM3/GD3, for example, is a melanoma-associated antigen involved in metastasis [3–5]. On the other hand, glucosylceramide (GlcCer) expression is associated with multidrug resistance in many cancer cells [6–8].

GSL are predominantly located at the plasma membrane and the early endosomes of the Golgi complex. In the plasma membrane, it has recently been shown that sphingolipid-derived molecules aggregate and form a less fluid and more ordered phase, referred to as membrane rafts, which are formed in the Golgi compartment and targeted to the plasma membrane. Rafts are considered to be small, mobile lateral assemblies of sphingolipids, particularly enriched in sphingomyelin and cholesterol, but also containing ceramide and GPI-anchored proteins. They have important roles in concentrating and modulating specific signaling molecules, such as Src-tyrosine kinase family members, growth receptors, and death receptors [9–12]. The role of rafts will not be discussed here as it has been the subject of recent excellent reviews [9,13]. Less complex sphingolipid-derived molecules, including ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate (S1P), are known signaling molecules in diverse receptor and non-receptor-mediated signaling pathways. These bioactive lipid mediators are formed as a result of stimuli-induced metabolism of complex sphingolipids. Ceramide has mainly been implicated in signaling pathways leading to suppression of growth, cellular senescence, differentiation, and apoptosis, whereas ceramide-1-phosphate mediates cell survival and is involved in synaptic vesicular fusion in neuronal cells, as well as neutrophil phagolysosome formation [14]. S1P has many biological actions and, importantly, acts counter to ceramide to mediate cell growth and survival, as well as influencing directed cell movement [15,16]. The biological effects of sphingosine may vary among cell types but it has been associated with negative effects on cell growth and survival and has been implicated as an inhibitor of protein kinase C and other protein kinases [17,18].

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Biosynthesis and structure of glycosphingolipids

The *de novo* biosynthesis of GSL is initiated at the cytosolic surface of the endoplasmic reticulum (ER) by the condensation of L-serine and palmitoyl coenzyme A to form 3-ketosphinganine catalyzed by serine palmitoyltransferase (SPT), a pyridoxal phosphate-dependent enzyme [19,20]. SPT has lower activity

than the other enzymes involved in biosynthesis and is rate-limiting and seems to be a key enzyme controlling cellular sphingolipid content. In the ensuing NADPH-dependent reaction, 3-ketosphinganine is reduced to D-*erythro*-sphinganine by 3-ketosphinganine reductase. The enzyme sphinganine-*N*-acyltransferase (ceramide synthase) transfers a long-chain fatty acid to the amino group of 3-ketosphinganine, resulting in the formation of D-*erythro*-dihydroceramide. The latter enzyme shows selectivity for stearic acid and is also able to acylate sphingosine derived from the "salvage pathway" of sphingolipid catabolism [21]. A double bond is then introduced between carbon atoms 4 and 5 by a desaturase to form ceramide [22]. All four enzymes of ceramide biosynthesis are located at the cytosolic surface of the ER membrane [23,24]. Recently, major progress has been made in cloning the enzymes of the *de novo* pathway.

Ceramide is a precursor of both GSL and sphingomyelin. In the synthesis of sphingomyelin, a phosphocholine group is transferred from phosphatidylcholine to ceramide. Sphingomyelin synthesis occurs in several cellular compartments, although most is synthesized on the lumenal side of the Golgi complex [25–27]. In vertebrates, GSL synthesis is initiated by coupling a glucose [28] or galactose [29] residue in a β-glycosidic linkage to the C1-hydroxyl of ceramide. Specific glycosyltransferases catalyze [30,31] the transfer of additional single nucleotide activated sugars onto ceramide forming more complex GSL (Figure 1) [30–32]. Most of the GSL of vertebrates arise from glucosylation rather than galactosylation of ceramide. Glucosylation is rate-limiting for ganglioside biosynthesis. This glucosyltransferase seems to be crucial during embryogenesis as the knock out of the respective gene has been shown to be lethal [33].

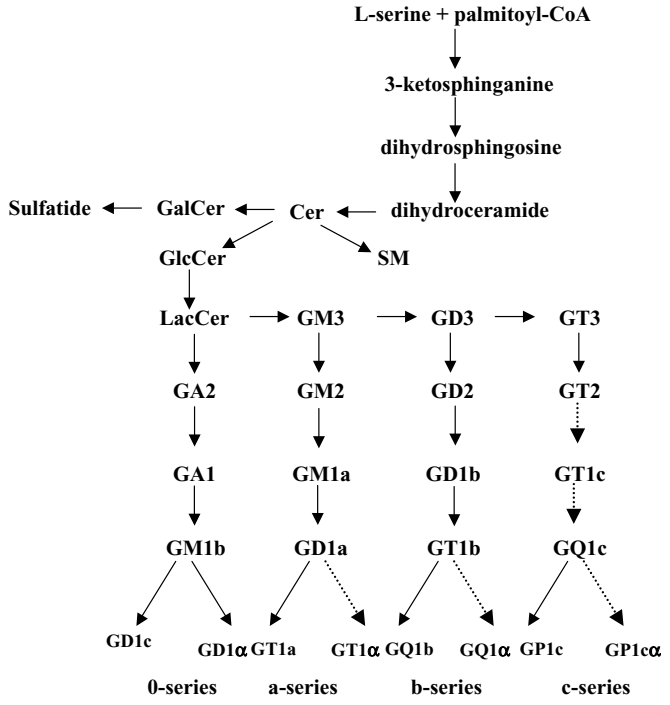

Figure 1. Simplified scheme of glycosphingolipid biosynthesis. For detailed descriptions of the biosynthetic pathways, see [38] and text.

Regarding topology of GSL synthesis, ceramide must be transported from the ER to the Golgi complex where it is glucosylated on the cytosolic surface of the Golgi-compartment [30,34–36]. Galactosylation of ceramide in the formation of glycosphingolipids of the galacto-series has been localized to the ER and Golgi. Transfer of a sulfate headgroup results in formation of sulfatides. The galacto-series gangliosides are found predominantly in the nervous system where they are important in development and normal functioning of the CNS [37–39]. Transport of ceramide from the ER to Golgi can occur by means of vesicular and non-vesicular mechanisms [40–42] and subsequent addition of sugar residues occurs on the lumenal face of the Golgi catalyzed by distinct glycosyltransferases [43].

Almost all gangliosides are structurally and biosynthetically derived from lactosylceramide which is formed by the transfer of a galactosyl residue to glucosylceramide. Sequential addition of one, two or three sialic acids to lactosylceramide results in formation of GM3, GD3 and GT3, respectively, precursors for more complex ganglio-series gangliosides. Sphingolipids are targeted to their cellular sites by both vesicular and non-vesicular trafficking [44].

Mechanisms of GD3-induced apoptosis

Ceramide is a well-known participant in the progression of pro-apoptotic signals initiated by Fas and tumor necrosis factor-α (TNF-α) through activation of their respective death receptors. The mitochondria also has a central role in ceramide-mediated cell death [45,46]. Generation of ceramide at the mitochondria, but not at other organelles, was shown to be involved in apoptosis of MCF-7 human breast cancer cells [47]. Fas cross-linking, TNF-α, and cell-permeable ceramide analogs all induce transient intracellular ceramide accumulation. An elegant study has shown that the intracellular ceramide accumulated due to Fas cross-linking is rapidly converted to GD3 by enhanced GD3 synthase activity in lymphoid and myeloid cell lines [48]. Antisense RNA against GD3 synthase prevents apoptosis, implying the need for newly-synthesized GD3. On the other hand, enforced expression of GD3 synthase was sufficient to trigger apoptosis. In this study, other gangliosides, such as GD1a, GT1b or GM1, failed to mimic GD3-induced cell killing [48]. Use of broad-spectrum caspase inhibitors revealed that caspases upstream of GD3 synthesis were crucial for Fas-induced apoptosis, suggesting modulatory interaction between GD3 and caspases. With respect to the mechanism by which GD3 induces apoptosis, changes in the mitochondrial membrane potential ($\Delta\Psi_m$) and increased reactive oxygen species (ROS) production have been demonstrated [48–51]. As a consequence of decreased $\Delta\Psi_m$, permeability of the inner mitochondrial membrane increases, causing the collapse of the ion gradient along the membrane and depolarization of the

mitochondria. ROS have been implicated in the initiation phase as well as in the execution phase of the apoptotic program, depending on cell type. Also, addition of ROS or depletion of endogenous antioxidants induces apoptosis that can be reversed by exogenous addition of antioxidants. The detailed mechanisms by which ROS function in cell death are not yet clear, but they are likely to be involved in activation of executionary caspases. In TNF-α-resistant hepatocytes, it was demonstrated that TNF-α was still able to induce synthesis of GD3. Only after depletion of mitochondrial glutathione, which is crucial for the maintenance of the cellular redox state, were cells sensitized to TNF-α- or GD3-mediated apoptosis [52]. This study underlines the importance of oxidative stress in TNF-α-mediated apoptosis in hepatocytes. By using inhibitors or antioxidants, it was shown that GD3 interacts with complex III of the mitochondrial electron transport chain causing an oxidative burst that precedes mitochondrial swelling. Cytochrome c and apoptosis inducing factor (AIF) are subsequently released, leading to activation of the caspase cascade and eventual DNA fragmentation [50,51]. *In vitro* studies performed with isolated mitochondria revealed that short-chain ceramides and glycosphingolipids, such as GlcCer, LacCer, GD1a, and GM1, are able to mimic GD3, while sphingosine and sphinganine failed to do so. Apparently, the *N*-acylsphingosine (ceramide) moiety is required for interaction of GD3 with the mitochondria rather than the carbohydrate component [50]. However, other groups showed that GD3-mediated effects on isolated mitochondria are very specific and could not be mimicked by C_2-ceramide, GM1, GM3, GD1a, or GT1b [53,54]. Future studies are needed to clarify this discrepancy and to determine the minimal structural requirement that enables GD3 to interact with and recruit mitochondria to the apoptotic signal transduction pathway.

GD3 can directly activate $\Delta\Psi_m$ independently of Ca^{2+}, although Ca^{2+} has been shown to act synergistically with GD3 [53]. It has been suggested that the effects of GD3 on mitochondria are mediated by the opening of the mitochondrial permeability transition pore (MTP), rather than by inhibition of the respiratory complex, as GD3-mediated effects could be prevented with the MTP blocker, cyclosporin A [54]. The MTP is a conductance channel formed by several different proteins, which is inserted into the mitochondrial membrane [55].

Bcl-2 is a proto-oncogene known to suppress cell death by diverse stimuli [55]. One mechanism by which Bcl-2 protects cells is suppression of the formation of ROS by acting as an antioxidant [55,56]. Because several studies have shown that ROS production in the mitochondria is a key target for apoptogenic GD3, it is conceivable that Bcl-2 might be able to modulate this pathway as well. Indeed, in T cell lymphoma CEM cells stably overexpressing Bcl-2, GD3 failed to induce mitochondrial changes or release of cytochrome c, AIF and activate caspase-9 [51]. Similar observations were made in oligodendrocytes where GD3-induced increase in $\Delta\Psi_m$ and cytochrome c release could be partially blocked by enforced Bcl-2 expression [57]. How Bcl-2 blocks GD3-induced cell death is not known yet. Several models have been proposed to explain how Bcl-2 might exert its anti-apoptotic function. It could prevent pore formation induced by other pro-apoptotic Bcl-2 family members, such as Bax/Bak, via increased heterodimerization of these proteins, or it could inhibit the opening of the MTP [58,59]. Moreover, Bcl-2 family proteins appear to regulate voltage-dependent anion channel (VDAC) function [60,61]. Further studies are needed to identify the relevant GD3 targets which are under Bcl-2 control. However, pretreatment of isolated mitochondria with cyclosporin completely suppressed GD3-induced swelling and release of apoptogenic factors, indicating that GD3 acts at the level of the MTP. Whether this is due to a direct interaction with any of the MTP components remains to be established [51].

Ceramide can be generated from degradation of sphingomyelin by either acidic sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase). Alternatively, ceramide generated by *de novo* synthesis has also recently been implicated in apoptosis [62]. Furthermore, it has been reported that ceramide generated by aSMase, and not nSMase, is involved in Fas and TNF-α signaling pathways activated in GD3 mediated cell death even though nSMase is active and contributes to the increase in ceramide in human colon cells [63]. These results were further confirmed with Niemann-Pick-derived lymphoblastoid cells that are devoid of aSMase but display normal nSMase activity [64]. In these cells, Fas failed to initiate the apoptotic program. Reconstitution of aSMase activity or addition of exogenous aSMase, however, caused GD3 accumulation and efficiently triggered the apoptotic program after Fas cross-linking or γ-irradiation.

Gangliosides are distributed predominantly on the plasma membrane and in the early Golgi compartment where they are synthesized. GD3 synthase (α2,8-sialyltransferase), which resides in the Golgi, adds a second sialic acid to GM3 to produce GD3. Just as is the case with ceramide, there seems to be a dichotomy in the signaling properties between newly synthesized GD3 and GD3 formed from degradation of other complex gangliosides. It appears in this case that newly-synthesized GD3 is involved in regulating apoptosis [65]. The question arises as to how newly formed GD3 is targeted to mitochondria where it executes its function in cell death. Different pathways might come into play. First, mitochondria might be in close physical contact with the ER/early Golgi to form a functionally interconnected network which has been described recently [66–68]. Second, and more likely, GD3 might be redistributed to mitochondria by actin-dependent endosomal vesicles. Indeed, disruption of actin cytoskeletal organization prevents release of GD3 from plasma membrane and co-localization with mitochondria [65]. Also, GD3 was shown to co-localize and associate with the actin cytoskeletal protein ezrin upon Fas cross-linking [69]. Moreover, pretreatment of cells with inhibitors of vesicular transport, such as monensin or mannose-6-phosphate, abolished localization of GD3 with mitochondria in hepatocytes treated with TNF-α [65]. Trafficking of GD3 was monitored over time and co-localization of GD3 was seen with markers specific for

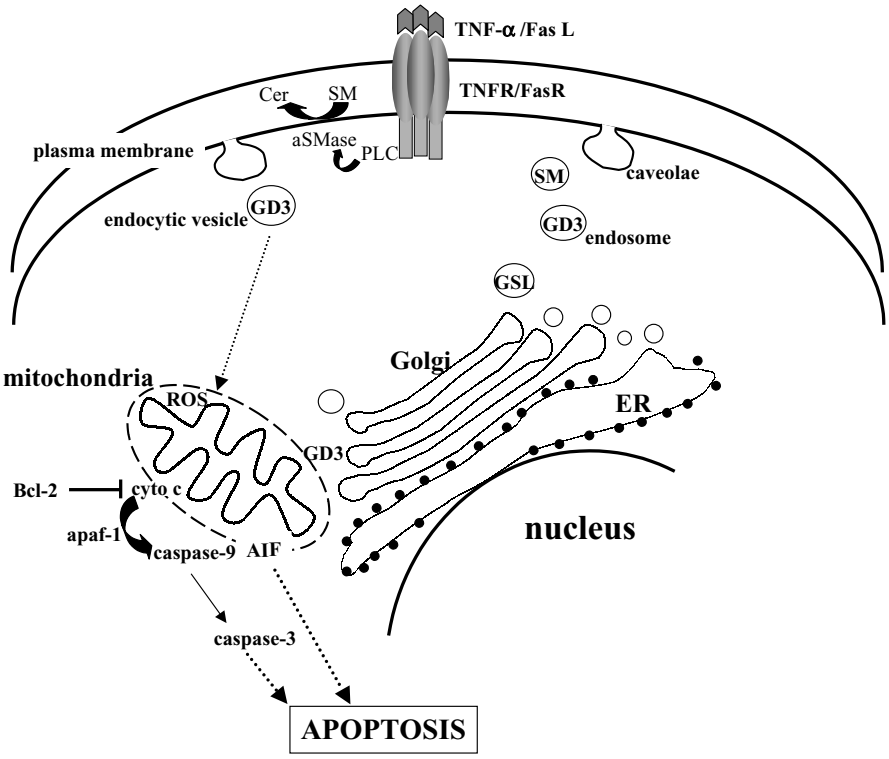

Figure 2. Signaling pathway of GD3-induced apoptosis. After oligomerization of the Fas or TNF-α receptors, aSMase is activated in a PC-PLC-dependent manner. Ceramide accumulates and activates GD3 synthesis. GD3 is targeted from the plasma membrane by vesicle transport or alternatively by physical redistribution from the Golgi to the mitochondria. There, GD3 perturbs the mitochondrial membrane leading to release of cytochrome c and AIF and caspase-9 activation, which activate the execution phase of the apoptotic program leading to demise of the cell.

plasma membrane, early endosomes, late endosomes, and finally with mitochondria [65]. GD3 ganglioside on the plasma membrane is localized, most likely, in specialized rafts, known as caveolae, where it can be internalized through endocytosis and trafficked to mitochondria (Figure 2). Co-localization of GD3 with caveolin-1 has been described previously [70].

As mentioned above, only ceramide generated in specific compartments, such as mitochondria or at the plasma membrane, has been shown to be involved in programmed cell death [47,71,72]. Furthermore, depending on cell type and/or agonist, aSMase and/or nSMase contribute to ceramide generation. Cells derived from aSMase null mice are defective in Fas-, radiation-, and TNF-α-induced cell death [63,64,73,74]. aSMase is active mainly in acidic compartments, such as recycling endosomes, and soluble aSMase is taken up by endocytosis and transported to acidic compartments. Membrane-bound forms of aSMase have also been detected in caveolae microdomains enriched in sphingomyelin that can be activated by various stimuli resulting in formation of ceramide [75,76]. Translocation of aSMase from intracellular compartments to plasma membrane rafts has been demonstrated after Fas stimulation [77].

Two different cytoplasmic domains have been described in the 55 kDa TNF receptor. One domain is able to activate nSMase and the other activates aSMase with no apparent crosstalk between them. The aSMase activation domain resides in the so-called death domain of the TNF receptor responsible for the cytotoxicity of TNF-α [78]. A phosphatidylcholine-specific phospholipase C activity was also required for aSMase activation [79,80].

It is assumed that intracellular ceramide concentrations regulate sphingolipid and glycosphingolipid metabolism; and, hence, ceramide should be targeted to the Golgi complex. Because aSMase has been shown to reside in caveolae, decreased sphingomyelin and concomitant ceramide production can lead to structural changes of the plasma membrane which can somehow stimulate endocytosis and trafficking of ceramide to the ER and Golgi, thereby enhancing GD3 synthesis. This might also explain why ceramide generated via aSMase, but not nSMase, is able to activate *de novo* GD3 synthesis.

Glycosphingolipids with anti-apoptotic properties

Difficulties in effective chemotherapy correlate with defective activation of programmed cell death on several distinct levels in many types of tumors [81]. Chemotherapeutic agents often exert some of their effects through generation of ceramide even though their mechanisms of action might differ.

Some stimulate *de novo* ceramide synthesis whereas others induce sphingomyelin hydrolysis or block ceramide degradation. Multidrug resistance, defined as cross-resistance to a variety of chemotherapeutic substances, is a common phenomenon in the treatment of various cancers. Besides accelerated removal of the drug (*i.e.* enhanced drug efflux via the P-glycoprotein pump), their intracellular effects may be altered [82,83]. It is also possible that multidrug resistance could result from modulation of ceramide metabolism whereby ceramide accumulation is prevented or it is converted to less toxic molecules [6,7,84]. Yet another way to keep endogenous ceramide low is by GlcCer synthase catalyzed conversion to GlcCer which has been shown to have growth stimulatory and anti-apoptotic effects [85]. Studies with exogenous administration of GlcCer revealed that it is able to stimulate growth of keratinocytes even in aged murine epidermis where epidermal growth is normally reduced [86]. Furthermore, GlcCer is consistently increased in several multidrug-resistant cancer cell lines [83,87]. In this regard, it was demonstrated that some sensitive cells acquire drug resistance by overexpressing GlcCer synthase [88]. Conversely, blocking glycosylation of ceramide with different agents, such as verapamil, tamoxifen, cyclosporin A or PDMP, in multidrug resistant MCF-7 breast cancer cells, sensitized them to adriamycin [89]. In addition, GlcCer synthase antisense RNA rendered otherwise resistant cells sensitive to drug treatment [88–91]. Reduced tumorigenicity and metastatic potential of melanoma cells was also observed *in vivo* with GlcCer synthase antisense RNA [92].

Another ganglioside that has been implicated in protection of cells from apoptosis is the monosialylganglioside GM1. GM1 has been shown to prevent apoptotic cell death in growth factor-deprived neuronal PC12 cells [93]. GM1 acts by promoting nerve growth factor (NGF)-induced TrkA dimerization. It has also been demonstrated that NGF signaling can activate sphingosine kinase to form S1P that acts as a pro-survival signal [93,94]. Similar results were obtained in a study conducted in rat heart fibroblasts where GM1 was shown to act like S1P and protect cells from C_2-ceramide or staurosporine-induced cell death [95]. It was also demonstrated in this study that GM1 enhanced S1P production by activating sphingosine kinase [95]. In a more physiologically relevant study, application of GM1 also protected the mouse heart from hypoxic cell death. Again, sphingosine kinase-dependent activation by protein kinase Cε was suggested [96]. These results have relevance to human physiology and there are ongoing clinical trials using GM1 ganglioside as a therapeutic agent for promoting nerve regeneration in Alzheimer's disease [97]. Furthermore, autoantibodies against various glycosphingolipids have been detected in patients with different neurological disorders, and have been suggested to play a critical role in development of diseases of the nervous system. In contrast, Le(y) antigen expression is correlated with apoptosis [98].

Gangliosides can also regulate cell signaling by altering growth factor receptor functions [99]. High GM3 ganglioside expression on keratinocytes has been correlated with inhibited cell growth and low expression has been reported in several hyperproliferative skin disorders, including psoriasis and squamous cell carcinoma [100,101] where programmed cell death is aberrant. Ganglioside GM3 was shown to interfere with binding of EGF and activation of its receptor which is required for proliferation [102].

Surprisingly, GM3 is also pro-apoptotic in certain types of cells, particularly in the presence of metastasis-suppressing gene product CD82 and its analogue CD9. It was shown that the malignancy-suppressing effect of CD82 or CD9 is based partially on cell motility inhibition and apoptosis induction promoted by concurrent GM3 synthesis and N-glycosylation [103]. GM3 in various colorectal carcinomas may also promote apoptosis, since enhancement of endogenous sialidase promotes tumor malignancy and metastasis through inhibition of Bcl-2 [104]. These dual actions of GM3 merit further study.

Significance of gangliosides in pathological processes

It has long been known that tumor cells display a different pattern of cell surface glycosphingolipids than corresponding untransformed cells [1,105]. Predominant expression of specific gangliosides, GD3, GM2, or GD2, has been observed on several types of tumor cells including melanoma, neuroblastoma, lymphoma, and ovarian cancer cells [105,106]. Thus, antibodies against specific gangliosides have received consideration as immunotherapeutic agents and clinical trials have been initiated [4,106–108].

Augmented GSL shedding, which is the release of cell surface components, is a characteristic of cancer cells. Shedding seems to be important for infiltration and metastasis of the tumor as well as for suppression of the immune system [109]. The underlying mechanism by which the released components evoke these biological effects is not yet fully understood. Gangliosides are among the main constituents of the released molecules. *In vitro,* and more importantly, *in vivo* effects of gangliosides shed from T cell lymphoma on bone marrow cells (BMC) have been documented [110]. These gangliosides not only impaired cell viability but also induced apoptosis of BMC. The shed gangliosides activated NF-κB and elevated expression of p53 and Bax, both of which have been described as components of pro-apoptotic signaling pathways. The apoptosis effects were ascribed to GD3 by investigations with antibodies against the major ganglioside species produced by T cell lymphomas. GD3 exogenously applied to BMC effectively induced apoptosis, further confirming this finding [110].

Another disease in which GD3 seem to be involved in is the progression of pathogenesis in Farber Disease, a lysosomal storage disorder which results from an acid ceramidase deficiency. As a consequence, ceramide accumulates in lysosomes leading to tissue damage, although the detailed mechanisms of tissue destruction are not well known. Histochemical analyses of tissues from affected patients revealed a high apoptotic rate

that correlated with concomitant elevated levels of GD3 and activated caspase-3 [111].

Conclusions

There is now abundant evidence documenting the importance of sphingolipid-derived signaling molecules. Although some sphingolipids have been well established as second messengers, *i.e.* ceramide, sphingosine and S1P, others await more detailed investigations. One difficulty in identifying the specific sphingolipid involved in a particular signaling pathway is their complex interconversion. For example, functions attributed to sphingosine might actually result from its conversion to S1P. Moreover, some effects of ceramide might result from its conversion to ceramide-1-phosphate, GlcCer, or even sphingosine and S1P. Also rapid degradation of gangliosides to ceramide has been described and this might confuse distinctions between ceramide and ganglioside-mediated effects [112]. As found for GM1, one GSL might also be able to stimulate the generation of another sphingolipid [95]. Since GD3 has mainly been associated with cell death and GM1 with survival, it will be very important to determine exactly what structural feature of these molecules is required for initiation of particular signaling pathways. This further emphasizes how important it is to elucidate the mechanism by which a molecule produces a certain biological response in order to design drugs for therapeutic applications.

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Modulation of adpative immune responses by sphingosine-1-phosphate

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Abstract

Sphingosine-1-phosphate (S1P) has long been recognized as a mediator of a variety of cell functions. A growing body of evidence has accumulated demonstrating its role in cell migration and as a mediator of growth factor-induced events. In recent years, it has become apparent that S1P also mediates many cytokine and chemokine functions. Cells of the immune system function and migrate in response to a complex network of cytokines and chemokines, and the outcome is determined by the interplay of the effects of these molecules on the target cell. S1P may be a bona fide component of these networks and influence the responses of cells to these immune modulators.

Keywords: Cytokines; Chemokines; GPCR; FTY720; Sphingosine-1-phosphate

1. Introduction

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite formed by the phosphorylation of sphingosine catalyzed by two isoforms of sphingosine kinase (SphK), is a multifunctional mediator of a variety of cell processes important for immune system functions, including cell growth regulation, inhibition of apoptosis, vascular development, and migration. S1P is an extracellular component of plasma and tissue fluids and functions by binding to a family of five specific cell surface G protein-coupled receptors. It is synthesized by many types of cells in response to a variety of stimuli and may also act as an intracellular second messenger. The extracellular and intracellular functions of S1P have been the subject of recent reviews [1–3] and will not be discussed extensively here. Interesting recent findings have begun to define a pivotal role of S1P in immunology as a modulator of cytokine synthesis as well as cellular responses to some chemokines. S1P may also function as a downstream mediator of cytokine signaling. Because cytokines and chemokines play central roles in homeostasis and activation and regulation of the immune system, S1P is emerging as an important player in the control of immune responses. The recent findings that the immunomodulatory drug, FTY720, when phosphorylated, is a S1P mimetic that acts by binding to S1P receptors [4,5], thereby mediating profound effects on the immune system, has highlighted the role of S1P and its receptors in chemokine and cytokine functions in the immune system.

2. Cytokine signaling through S1P

Over the past 15 years, a mass of evidence has accumulated that demonstrates that sphingolipid metabolites, particularly ceramide, act as second messengers and mediate many of the biological effects induced by TNF-α [6–9]. During inflammatory and other immune responses, TNF-α activates endothelial cells by inducing the expression of adhesion molecules, such as E-selectin and vascular cell adhesion molecule-1 (VCAM-1), and stimulates secretion of various cytokines. In human umbilical vein endothelial cells (HUVEC), the SphK inhibitor, *N*,*N*-dimethylsphingosine (DMS), inhibited TNF-α-induced upregulated expression of adhesion molecules, as well as activation of ERK and NFκB, leading to the conclusion that formation of S1P was critical for these TNF-α-mediated events [10]. Similarly, increased S1P levels in human neutrophils induced by TNF-α has been linked to TNF-α-induced priming of these cells [11,12].

TRAF2, a component of the TNF receptor complex that is important for NFκB activation and anti-apoptosis [13,14], may be the link between TNF-α and SphK activation [15]. Co-immunoprecipitation studies demonstrated physical interaction between TRAF2 and SphK1, and transfection with dominant-negative TRAF2 blocked TNF-α-stimulated SphK activity, suggesting that TRAF2 is a scaffolding protein that brings the TNF-α receptor and SphK together [15]. Several

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groups have reported that inhibition of apoptosis by TNF-α is mediated by SphK activation and S1P generation [16,17] and it has been found that a catalytically inactive SphK abolishes the cytoprotective effects of TRAF2, suggesting that SphK activity is required for TRAF2 to function, and therefore also for the anti-apoptotic effects of TNF-α [15]. In human hepatocytes, inhibition of SphK with DMS indicates that not only SphK but also NF-κB and PI3 kinase/Akt are involved in signaling pathways for protection from the apoptotic actions of TNF-α [17]. However, it is still not clear in this case whether S1P acts intracellularly to activate PI3 kinase or extracellularly via S1P receptors.

Because DMS and other pharmacological SphK inhibitors inhibit both SphK1 and SphK2 and also may have other non-specific effects, it was important to confirm the importance of SphK by more specific approaches. To further address the role of SphK and S1P formation in TNF-α-mediated inflammatory events, Pettus et al. [18] used a more specific molecular approach to downregulate SphK1 and SphK2 expression. They found that in L-929 fibroblasts, small interfering RNA (siRNA) targeted to SphK1, but not SphK2, decreased SphK activity and concomitantly inhibited the stimulatory effects of TNF-α on COX-2 expression and secretion of the inflammatory mediator prostaglandin E_2. These studies clearly place SphK1 and S1P formation in the TNF-α signaling repertoire (Fig. 1). However, links between the TNF receptors and SphK, as well as the downstream targets of S1P, are not yet well-characterized.

Interestingly, a physical association between Sphk2 and the β chain of the IL-12 receptor has recently been demonstrated [19] and Sphk2 augments IL-12 receptor signaling, further indicating a role for S1P in Th1 responses and inflammation.

Another protein that has been gaining more attention and has recently been linked to S1P is the tumor-associated cytokine, autotaxin, a protein factor that increases tumorigenicity and metastasis [20,21]. Autotaxin is an ectoenzyme originally shown to be a phosphodiesterase with important functions in non-transformed cells, such as oligodendrocytes during myelination [22]. Autotaxin has recently been discovered to have lysophospholipase D (lyso-PLD) activity that can generate lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) [23,24], as well as S1P from sphingosylphosphorylcholine (SPC) [25]. It is therefore possible that some of the biological effects of autotaxin are mediated through the generation of S1P and/or LPA in the extracellular environment. Indeed, treatment of cells with SPC and autotaxin mimics some of the effects of S1P, such as increased motility and angiogenesis [25].

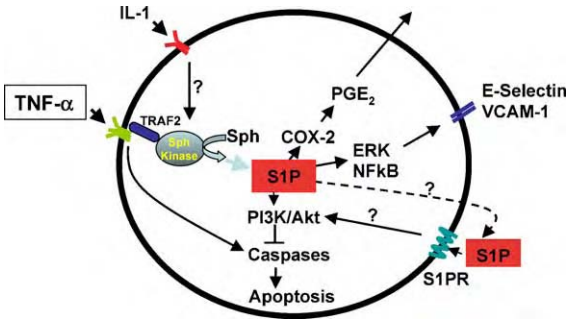

Fig. 1. The role of S1P as a mediator of TNF-α receptor signaling.

3. S1P Effects on cytokine production

Although there is substantial evidence establishing S1P as an extracellular mediator of cell migration and differentiation, the role of S1P in the control of cytokine and chemokine production and as a mediator of their effects is an emerging area of research. Cytokines and chemokines play central roles in activation, differentiation into effector states, function and migration of immune cells, including T- and B-lymphocytes, monocytes and dendritic cells [26,27]. T cells express S1P receptors, particularly $S1P_1$ and $S1P_4$, and nanomolar concentrations of S1P stimulate chemotaxis of Jurkat and splenic T cells [28]. Given that S1P and cytokines/chemokines have similar roles in different cell types, it might be expected that in immune cells, their signaling pathways would communicate and influence each other.

There are conflicting reports of the effects of S1P on the production of IL-2 and IFN-γ in activated T cells. Splenic CD4 T cells secrete IL-2, IFN-γ and IL-4 when stimulated with anti-CD3 together with anti-CD28. Co-treatment with low concentrations of S1P decreased IFN-γ and IL-4 secretion, but not IL-2 [28]. A similar pattern of inhibition of cytokine secretion by S1P was seen when the cells were stimulated with anti-CD3 and IL-7, but in this case, S1P marginally inhibited IL-2, although nanomolar concentrations were not effective. Similar doses of LPA also inhibited IL-2 secretion, in agreement with previous reports [29,30]. On the other hand, S1P enhanced IFN-γ and IL-2 secretion in CD3/CD28-stimulated peripheral blood lymphocytes [31]. There were several differences between these studies that may account for the discrepancy. The former study used purified CD4-positive splenic T cells and S1P concentration less than 1 μM, whereas the latter study utilized peripheral blood T cells, a mixture of both CD4 and CD8 cells, and S1P at much higher concentrations. In addition, it is possible that the effects of low concentrations of S1P are mediated through distinct cell surface S1P receptors while S1P may act intracellularly at higher concentrations. Nevertheless, S1P can alter the cytokine profile in activated T cells (Fig. 2).

The cytokine and chemokine expression profiles of activated T cells are determined by the interactions with antigen presenting cells (dendritic cells (DC), in particular) that sense environmental “cues” and then secrete cytokines that determine whether Th1 (cell mediated) or Th2 (antibody) responses occur. DC are specialized antigen-sensing and presentation cells that are important in the activation of T and

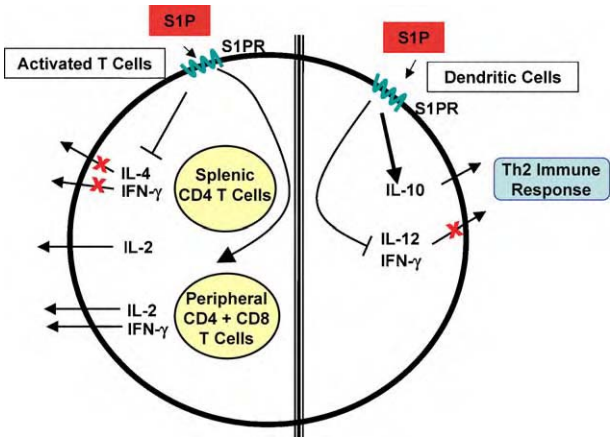

Fig. 2. The effect of extracellular S1P on secretion of cytokines by dendritic cells and activated T cells.

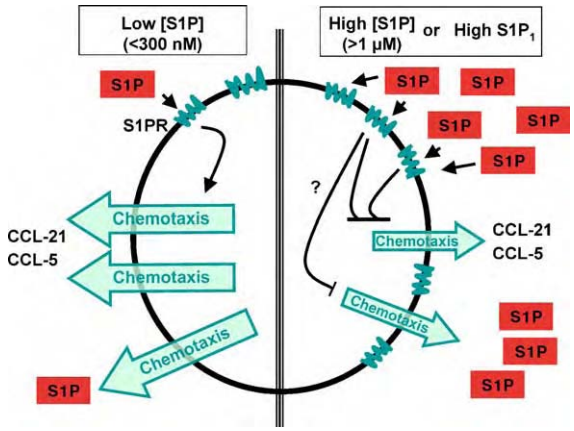

Fig. 3. The effect of varying S1P levels and $S1P_1$ expression on the chemotactic response of T cells to the chemokines CCL-21 and CCL-5.

B cells [32,33]. Immature DC reside in peripheral tissues where they capture and process antigen. This initiates their maturation and migration to secondary lymphoid tissues where they stimulate T and B cells to initiate the immune response to the antigen. DC maturation, as well as lymphocyte stimulation, requires a variety of cytokines and the composition of the cytokine environment during maturation and stimulation can determine the nature of the immune response. Although S1P does not affect basal cytokine expression in immature DC, it does alter the cytokine profiles of mature DCs [34]. In DC matured by treatment with lipopolysaccharide, S1P inhibited secretion of IL-12 and IFN-γ, but enhanced secretion of IL-10 (Fig. 2). IL-10 promotes a Th2 type immune response whereas it inhibits a Th1 response. Indeed, when S1P-treated DC were used to prime naive T cells, the T cells displayed an enhanced Th2 response, characterized by enhanced IL-4 and decreased IFN-γ production. In contrast, naive T cells that were primed with untreated DC displayed a predominantly Th1 profile, characterized by enhanced IFN-γ production [34].

Chemokines are a large group of secreted, small chemotactic cytokines that control leukocyte (T cells, B cells, DC, macrophages/monocytes, granulocytes) traffic throughout the body [35]. Chemokines are loosely grouped into two categories: “homeostatic” chemokines, which control leukocyte movement during hematopoieis, their navigation through the lymph nodes, and thymic maturation and immune surveillance; and “inflammatory” chemokines, which recruit leukocytes to sites of inflammation and tissue injury.

Nanomolar concentrations of S1P stimulate chemotactic responses of CD4 and CD8 T cells, as well as immature dendritic cells [28,34]. Activation of CD4 T cells by TcR activation (anti-CD3 + anti-CD28) downregulates expression of $S1P_1$ and the cells lose their chemotactic responses to S1P, whereas their chemotactic responses to the chemokine CCL-21 are increased. CCL-21 is a homeostatic, secondary lymphoid tissue chemokine involved in splenic and lymph node T cell homing. Treatment of CD4 T cells with S1P concentrations within the normal plasma range, enhanced their chemotactic responses to CCL-21 and CCL-5 in transwell chamber motility assays [36–38]. However, treatment with micromolar concentrations of S1P inhibited chemokine-induced chemotaxis (Fig. 3). These studies were also carried over into an in vivo migration model in which a chemoattractant was injected into a dorsal subcutaneous air pouch in a mouse and fluorescent-labeled cells were injected intraperitoneally. The number of fluorescent cells that migrate into the air pouch is a measure of the chemotactic activity of the chemoattractant. Migration of T cells that were pretreated with micromolar concentrations of S1P into the air pouch was significantly lower into CCL-21-treated air pouches than migration of untreated T cells. In contrast to the effects of S1P on cytokines, S1P does not appear to affect CCL-21 and CCL-5 production, but only modulates responsiveness to these chemokines.

S1P is nearly as effective a chemoattractant for DC as are C5a and CCL19 [34]. Like T cells, DC express CCR7 and migrate in response to its ligands CCL19 and CCL21 [39,40]. Although S1P induces actin polymerization and chemotaxis in immature DC, these effects are not observed in LPS-induced mature DC [34]. Similarly, prion protein fragment 106-126 is a chemoattractant for monocyte-derived immature but not mature DC [41]. This chemotaxis was inhibited by blockade of SphK, suggesting that transactivation of S1P receptors may be involved in prion protein-induced motility of DC [41]. These observations could have implications for mediation of neuroinvasion in transmissible spongiform encephalopathies by circulating DC.

Interleukin-8 (IL-8) is an important chemoattractant that recruits neutrophils to sites of inflammation and is also a proangiogenic factor [42]. S1P increases IL-8 expression and secretion in a variety of ovarian cancer cell lines [42]. Bronchial epithelial cells also secrete IL-8 in response to stimulation by S1P, a process that is mediated by S1P-stimulated activation of phospholipase D (PLD)

and subsequent generation of phosphatidic acid and ERK activation [43,44].

Our lab has recently demonstrated that S1P induces production of chemokines by bone-marrow-derived mast cells (BMMC) [45]. Mast cells are key players in both immediate-type and inflammatory allergic reactions and nanomolar concentrations of S1P upregulated both mRNA and secretion of CCL-4 (M1P1β) and CCL-2 (MCP-1). CCL-4 and CCL-2 are inflammatory chemokines that induce the recruitment of inflammatory cells, such as monocytes and eosinophils, to sites of inflammation. Our results suggest that S1P may have a more significant role in the production of inflammatory chemokines than in regulating homeostatic chemokines, such as CCL-21. This enhancement of mast cell-mediated inflammation by S1P is consistent with its effects on DC, as S1P-treated DC promote Th2 responses that result in IgE production [34].

Our results also suggest that activation of SphKs, production of S1P, and consequently activation of S1PRs by FcεR1 triggering plays a crucial role in mast cell functions and might be involved in movement of mast cells to sites of inflammation [45]. We found that whereas transactivation of $S1P_1$ and Gi signaling are important for cytoskeletal rearrangements and migration of mast cells towards antigen, $S1P_1$ is dispensable for FcεRI-triggered degranulation. In contrast, $S1P_2$, whose expression is upregulated by FcεRI crosslinking, was required for degranulation and inhibited migration towards antigen [45].

S1P may also have a more indirect effect on functions of chemokines. Matrix metalloproteinases (MMPs) are a family of enzymes involved in extracellular matrix (ECM) remodeling during cell migration. S1P and MMPs appear to play complementary roles during cell migration, as S1P has been shown to upregulate MMP expression [46,47] and MMPs can also cleave and inactivate chemokines in the ECM. However, direct evidence for this interaction is lacking. Moreover, it has been reported that S1P induces MMP expression in endothelial cells and it also paradoxically antagonizes the cell surface proteolytic cleavage of pro-MMP, which is required for its activation [46].

$S1P_1$ and $S1P_4$ are the two predominantly expressed S1PRs on T cells [28]. During T-cell activation, $S1P_1$ expression decreases whereas chemotactic responses to CCL-21 increase. T-cell activation also reduced both the S1P enhancing (at low concentrations) and inhibitory (at high concentrations) effects on chemokine-induced chemotaxis [36]. Similarly, chemotactic responses to the chemokine CXCL-4 of rat hepatoma cells overexpressing $S1P_1$ were enhanced at low S1P concentrations and inhibited at micromolar S1P concentrations, while overexpression of $S1P_4$ did not influence these responses. In agreement, overexpressing $S1P_1$ in T cells produced stronger migratory response to low S1P concentrations [38]. However, nanomolar concentrations of S1P, which in naive T cells enhanced CCL-5 and CCL-21-induced chemotaxis, inhibited chemotaxis in the $S1P_1$ overexpressing cells (Fig. 3). These results suggest that increased expression of $S1P_1$ may have the same effects as high S1P levels on chemotactic responses to CCL-5 and CCL-21. As well, high $S1P_1$ expression may affect different chemokine responses differently; e.g. increased chemotaxis to CXCL-4 but decreased chemotaxis to CCL-21. Thus, the $S1P_1$ receptor appears to be the most important determinant of cytokine and chemokine functions that are regulated by S1P.

T-cell responses to S1P-induced chemokine effects are also determined by prior S1P exposure [37]. Pre-incubation for 1 h with 100 nM S1P abolished the subsequent inhibition by micromolar concentrations of S1P on CCL-21 chemotaxis and the enhancement by low S1P concentrations. However, by 24 h post pre-incubation, the cells recovered the inhibitory and enhancement effects of S1P. This may be a result of $S1P_1$ downregulation during the pretreatment and subsequent recovery of $S1P_1$ expression.

4. Phosphorylated FTY720 is a S1P receptor agonist

FTY720 is an immunosuppressive agent that is highly effective in the prevention of transplant rejection [48]. Its apparent mode of action is to sequester lymphocytes in secondary lymphoid organs, such as lymph nodes, without affecting T-cell activation and expansion [49,50]. As a result, FTY720 prevents lymphocyte invasion into the transplant, and therefore prolongs its survival, without the induction of general immunosuppression. More recently, it was shown that FTY720 can also prevent egress of mature T cells from the thymus [51]. FTY720 has structural homology to sphingosine, and there are now several reports demonstrating that FTY720 is phosphorylated in vivo and in vitro is a substrate for SphK2 [4,52,53]. The phosphorylated form of FTY720 is biologically active and binds to all of the S1P receptors except $S1P_2$ [4].

The mechanisms by which FTY720 (or phospho-FTY720) induces lymphocyte sequestration and inhibits egress from the thymus are not well-understood. Given that S1P affects lymphocyte migration, either directly or indirectly by altering cellular responses to chemokines and cytokines, and chemokines are important mediators of lymphocyte trafficking, it follows that FTY720 may function in a similar manner. Indeed, lymphocytes from mice treated with FTY720 showed enhanced chemotaxis to a number of chemokines, including CCL-21 and CCL-5 [54]. However, FTY720 did not induce significant differences in chemokine or chemokine receptor expression.

CCR7 is the receptor for CCL-21, and both are required for lymphocyte homing to lymph nodes. CCR7-null mice have decreased numbers of T cells in their lymph nodes, as do CCL-21-knockout (*plt*) mice. If CCL-21 is required for FTY720-induced homing and sequestration, it might be expected that a defect in CCL-21 production would antagonize the FTY720-induced accumulation of lymphocytes in secondary lymphoid tissues. Indeed, *plt* mice treated with

FTY720 for 6 or 12 h have higher numbers of lymphocytes in the periphery than wild-type mice, suggesting that CCL-21 is essential for homing in response to FTY720 [54]. However, in similar studies with CCR-7-deficient and *plt* mice, it was found that CCL19 and CCL21 chemokines do not play significant roles in FTY720-induced lymphocyte homing [55]. Treatment of CCR-$7^{-/-}$ and *plt* mice with FTY720 resulted in loss of lymphocytes from the peripheral blood with concomitant increased numbers in lymph nodes and Peyer's patches, albeit with a slower kinetics than those of wild type mice. The differences between these two studies may be that the time of exposure to FTY720 was not the same. Moreover, adoptively transferred CCR-7-deficient lymphocytes that were treated in vitro with FTY720 migrated into lymph nodes whereas CCR-7-deficient, untreated cells did not [55]. Although CCL-21 and CCR-7 do not appear to be required for FTY720 homing, CCL-21 does play some role in the accelerated lymphocyte homing.

The control of egress of mature T cells from the thymus is an important step in controlling the number of T cells in peripheral blood and secondary lymphoid organs. Migration of T cells through the thymus and subsequent egress is a complex process that is highly dependent upon chemokines. $S1P_1$-deficient thymocytes fail to leave the thymus, yet remain responsive to CCL-21. In addition, mature thymocytes from FTY720-treated wild-type mice fail to leave the thymus, yet remain responsive to CCL-21 [51]. It would therefore appear that chemokine-responsiveness of mature thymocytes is not affected by FTY720 or S1P in the same way as are peripheral blood and secondary lymphoid T cells. However, maturation and egress from the thymus involves interplay of multiple chemokines (reviewed in [35]) and FTY720 and/or S1P may influence the action of other chemokines involved in this process.

To date, FTY720, unlike S1P, has not been linked to any significant alterations in cytokine profiles that may specifically affect Th1 or Th2-type immune responses. In a Th1- versus Th2-mediated airway inflammation mouse model, it was shown that FTY720 effectively suppresses both responses, as well as suppressing both Th1 and Th2-associated cytokines in bronchial alveolar lavage fluid [56]. However, there is no evidence that FTY720 directly affected cytokine secretion and reduced cytokine levels in the alveolar lavage fluid could be a reflection of the reduced lymphocyte infiltration into the bronchial tissue.

5. Conclusion

From the studies cited in this review, it is clear that S1P plays a role in TNF-α signaling and that it can also act as an extracellular mediator to affect chemokine and cytokine functions. Cytokine and chemokine production, as well as the subsequent cellular responses, orchestrate a complex network of events that in the immune system result in the inflammatory responses of the innate immune system, selection of functional lymphocytes, deletion of self-reactive lymphocytes, cell activation and expansion in response to foreign antigen, and in dampening of the response after antigen clearance. Moreover, these events occur in different locations around the body and the movement of immune cells must be controlled so the proper cells will be in the correct site at the right time.

The data to date demonstrate that S1P may influence the direction of an immune response by modulating cytokine secretion patterns, as well as by modulating chemotactic responsiveness of DC and lymphocytes to chemokines. These latter events may enhance or inhibit immune cell migration into, or egress from tissues and organs. Lymphocytes express multiple S1P receptors that appear to have both unique and overlapping functions. Given that S1P receptor levels on lymphocytes vary depending on state of activation, and S1P levels may vary between plasma and tissues, the net effects of S1P on chemokine-responsiveness may be determined by the location and level of activation of the cell. For example, a naive T cell in the periphery may be responsive to the effects of S1P but once activated in a secondary lymphoid organ, it may lose its responsiveness not only to S1P but also to chemokines. However, this is an oversimplified view given the enormous complexity of chemokine and cytokine networks and the fact that studies to date have only investigated functions of S1P in the context of a few cytokines and chemokines. It is apparent that we have only barely scratched the surface of S1P and cytokine/chemokine networks.

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Appendix 5

PERGAMON

Progress in Lipid Research

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Review

Pleiotropic actions of sphingosine-1-phosphate

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that regulates diverse cellular responses including, growth, survival, cytoskeleton rearrangements and movement. S1P plays an important role during development, particularly in vascular maturation and has been implicated in pathophysiology of cancer, wound healing, and atherosclerosis. This review summarizes the evidence showing that signaling induced by S1P is complex and involves both intracellular and extracellular actions. The intracellular effects of S1P remain speculative awaiting the identification of specific targets whereas the extracellular effects of S1P are clearly mediated through the activation of five specific G protein coupled receptors, called $S1P_{1-5}$. Recent studies demonstrate that intracellular generated S1P can act in a paracrine or autocrine manner to activate its cell surface receptors.

Contents

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Nomenclature

AC	adenylyl cyclase
ASMC	aortic smooth muscle cells
CFTFR	cystic fibrosis transmembrane regulator
ER	endoplasmic reticulum
EDG	endothelium differentiation gene
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
HUVEC	human umbilical vein cells
JNK	c-Jun amino terminal kinase
LPA	lysophosphatidic acid
LPAR	lysophosphatidic acid receptor
NO	nitric oxide
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblasts
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin

S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor
SH3	Src homology 3
SPHK	sphingosine kinase
SPT	serine palmitoyl transferase
TM	transmembrane
VEGF	vascular endothelium growth factor
VEGFR	vascular endothelium growth factor receptor
VSMC	vascular smooth muscle cells

1. Introduction

The importance of the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P) as a regulator of many cellular functions has only been recognized within the last decade [1–5]. Zhang et al. in 1991 first demonstrated the importance of S1P in cell growth regulation [1]. Numerous studies have since shown that S1P is a potent mitogen for diverse cell types and also elicits various other biological effects including mobilization of intracellular calcium, regulation of cytoskeletal organization, differentiation, survival, and motility [6–9]. The discovery in 1998 that S1P is a ligand for cell surface G-protein-coupled receptors (GPCRs) [4] has accelerated studies in this area. This review will describe the synthesis and metabolism of S1P and discuss its biological actions.

2. Sphingosine-1-phosphate synthesis, metabolism and the sphingolipid rheostat model

S1P is a phosphorylated derivative of the long-chain sphingoid base sphingosine, which forms the backbone of all sphingolipids [10]. De novo synthesis of sphingolipids begins within the endoplasmic reticulum (ER) via the condensation of serine and palmitoyl coenzyme A, catalyzed by the pyridoxal-dependent enzyme, serine palmitoyl transferase (SPT), to form 3′-keto-sphinganine, which is then reduced to sphinganine (dihydrosphingosine). Dihydrosphingosine is subsequently *N*-acylated by ceramidase synthase, forming dihydroceramide. The introduction of the 4,5-*trans* double bond, converting the resulting dihydroceramide to ceramide, is catalyzed by dihydroceramide desaturase [11]. Ceramide is then converted to sphingomyelin and complex sphingolipids. Turnover of these ceramide-containing sphingolipids results in the formation of sphingosine and finally S1P by the respective action of ceramidase and sphingosine kinase (SPHK) (Fig. 1).

Sphingolipid metabolism is a dynamic process resulting in the formation of a number of bioactive metabolites including ceramide, ceramide-1-phosphate, sphingosine and S1P [9]. Sphingomyelin degradation occurs in lysosomes and endosomes and in the plasma membrane in response to growth factors, pro-inflammatory cytokines, arachidonic acid and cellular stresses. Following sphingomyelinase activation, sphingomyelin is hydrolyzed to ceramide, which is

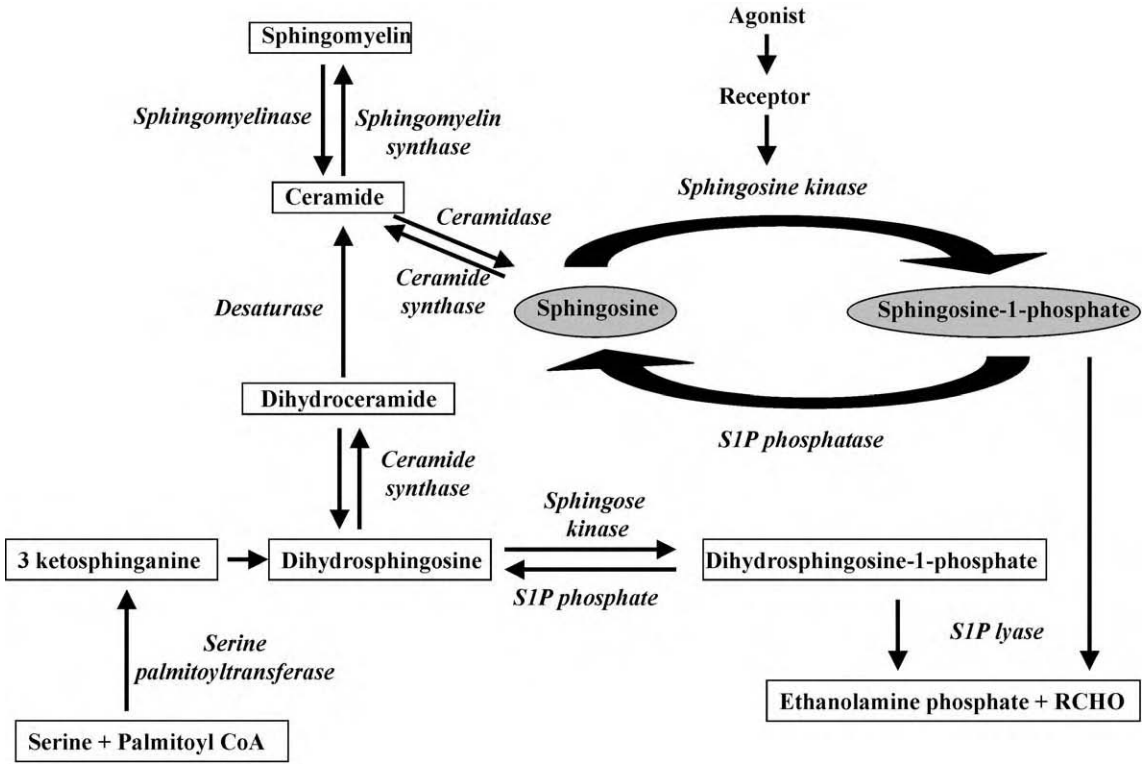

Fig. 1. Sphingosine-1-phosphate metabolism. De novo synthesis of sphingolipids begins in the ER with SPT catalyzed condensation of serine and palmitoyl transferase, forming dihydrosphingosine through the intermediate 3′ketosphinganine. Dihydrosphingosine is then converted to dihydroceramide by ceramide synthase which is subsequently converted to ceramide by dihydroceramide desaturase. Sphingosine and S1P are derived from ceramide by the sequential actions of ceramidase and SPHK. Additionally, turnover of stored sphingomyelin to ceramide, sphingosine and S1P by the sequential actions of sphingomyelinase, ceramidase and SPHK provides another source of S1P.

thought to be a critical regulator of cell growth arrest, differentiation and apoptosis [12,13]. Ceramidase catalyzes the deacylation of ceramide to produce a free fatty acid and sphingosine. Sphingosine has been shown to inhibit protein kinase C (PKC), and can also affect the activity of specific kinases [12,13]. As ceramide and sphingosine are usually associated with negative effects on cell growth and survival while S1P opposes these effects, the dynamic balance between the concentrations of these bioactive sphingolipid metabolites, the ''sphingolipid rheostat'', helps determine cell fate [14]. Curiously, there are some reports that S1P is pro-apoptotic, for example, in hepatic myofibroblasts [15], and it may either stimulate proliferation or induce apoptosis of mesangial cells, depending upon cell density [16]. Interestingly, radiation-induced oocyte loss, which is known to drive premature ovarian failure and infertility in female cancer patients, was completely prevented in adult female mice by in vivo treatment with S1P [17], providing a potentially important clinical application [18].

As discussed above, SPHK is the main enzyme responsible for S1P production. Two isoforms have been cloned, namely SPHK1 and SPHK2, which although highly homologous, differ in length and possibly function [19,20]. Total cellular SPHK activity is increased by many stimuli,

including growth factors, cytokines, phorbol esters and GPCR agonists, such as the muscarinic agonist, carbachol, and S1P itself [2,21]. The specific biological effects mediated by each of these SPHKs remain to be sorted out although some evidence has accumulated on the functions of SPHK1 in growth and survival. For example, overexpression of SPHK1 in NIH 3T3 fibroblasts increased S1P, decreased ceramide and increased stress survival [22,23]. Additionally, overexpression of a dominant negative form of SPHK1 in HEK 293 cells abolished TNF-α-stimulated S1P formation and p42/p44 MAPK activation [24]. Future studies should be focused on SPHK2 which is the predominant isoform in some types of cells [19,20].

Breakdown of S1P is catalyzed by a pyridoxal phosphate-dependent lyase located in the ER, which degrades S1P to phosphoethanolamine and palmitaldehyde, and by conversion back to sphingosine by a specific phosphatase (SPP-1) located in the ER [25,26]. Regulation of SPP-1 may also play a significant role in determining the relative intracellular levels of S1P and sphingosine and ceramide. Thus, overexpression of SPP-1 in NIH 3T3 cells decreased S1P levels by approximately 2-fold and increased ceramide levels 2-fold, whereas sphingosine levels were unchanged [27], suggesting that SPP-1 activity modulates intracellular concentrations of sphingolipids and the resultant dephosphorylated sphingoid bases are then rapidly metabolized to ceramide [26].

3. S1P secretion

Although the relative intracellular concentrations of the bioactive sphingolipid metabolites are determined mainly by the enzymes responsible for their generation and metabolism, the potential contributions of uptake and secretion remain less well defined. The S1P concentration in serum is relatively high where it is present in an albumin-bound form. It is thought to arise mainly from activated platelets, which produce and store it [28]. Accumulation of S1P within platelets has been attributed to their unique lack of the major S1P degradation enzyme, S1P lyase. Extracellular S1P may also be derived from other cell types, including mast cells and monocytes [29,30]. However, the precise mechanisms responsible for S1P release remain poorly characterized. Recent studies have suggested that extracellular S1P might also be derived from extracellular metabolism of sphingomyelin, since sphingomyelinase, ceramidase and SPHK have all been reported to be secreted by cells [31–33]. On the other hand, the cystic fibrosis transmembrane regulator (CFTR), a member of the ATP binding cassette family of proteins, may be involved in the uptake of extracellular S1P [34]. Uptake into cells could influence the balance between extracellular and intracellular S1P concentrations and also affect the ability of S1P to modulate biological activity via interactions with S1PRs. Dephosphorylation of extracellular S1P by ecto-phosphatases could also influence the concentration of S1P available to act on cell surface receptors [35].

4. S1PR-mediated effects of S1P

The extracellular effects of S1P have mainly been attributed to binding to five specific members of the Endothelial Differentiation Gene (EDG) family of GPCRs, now called $S1PR_{1–5}$ [9,36]. Three closely related yet distinct LPARs, $LPA_{1–3}$, specifically bind lysophosphatidic acid (LPA),

a bioactive phospholipid with similar biological effects and structure to S1P [37,38]. Interestingly, the LPAR genes contain an intron in the region encoding the 6th transmembrane domain (TM6) which is not present in S1PR genes [39]. These lysophospholipid receptors also are somewhat homologous with the cannabinoid receptor subfamily (<30%), perhaps suggestive of a possible common ancestral gene [40].

The amino-termini of the S1PRs are positioned toward the extracellular space whereas the C-termini are oriented intracellularly. The arrangement of the seven transmembrane domains forms a pocket that facilitates ligand binding. A modeling study of $S1P_1$ indicated that the basic amino acids R^{120} and R^{292} ion pair with the phosphate of S1P [41]. The S1PRs also all have a conserved anionic residue corresponding to E^{121} in $S1P_1$ that has been proposed to interact with the positively charged sphingoid base amino group [41]. In contrast, the LPARs have a neutral glutamine residue at this position which might be a docking site for the neutral hydroxyl group in LPA [41]. The C-terminus of $S1P_3$ is unique amongst the S1PRs in that it contains a putative class I Src homology 3 (SH3) interaction motif (RASPIQP), important in tyrosine kinase signaling. The tissue distribution, G-protein coupling and main signal transduction effects of the S1PRs are summarized in Table 1.

5. $S1P_1$

$S1P_1$ was originally identified as an early immediate gene product induced in phorbol ester-differentiated HUVECs and was the first S1PR to be cloned [42]. It is expressed in most mammalian tissues with highest expression in skeletal structures undergoing ossification, endothelial cells, and the Purkinje cell layer of the cerebellum. $S1P_1$ was also reported to be a low-affinity receptor for LPA [43]. However, subsequent studies with membranes from Sf9 cells co-expressing $S1P_1$ and G_{i2} failed to demonstrate an increased biological effect of LPA [44]. Others were also unable to detect competition of [^{32}P]S1P binding to $S1P_1$ by LPA [4, 45]. Moreover, LPA failed to significantly increase basal $S1P_1$ phosphorylation in CCL-39 hamster lung fibroblasts stably expressing $S1P_1$ [46].

$S1P_1$ signaling is involved in cell migration, formation of new blood vessels, and vascular maturation [5,9,36]. In fact, *s1p1* deletion was embryonic lethal and this was largely attributed to defective vessel maturation [47]. $S1P_1$ signaling via a $G_{i/0}$-coupled mechanism has been demonstrated

Table 1
The S1PR family[a]

Receptor	Tissue distribution	Coupled G-proteins	Signalling involved
$S1P_1$/EDG1	Widely distributed	$G_{i/0}$	↓AC,↑ERK,↑PLC,↑Akt,↑eNOS,↑Rac,↑Rho
$S1P_3$/EDG3	Widely distributed	$G_{i/0}$, $G_{q/12/13}$	↓AC,↑ERK,↑PLC,↑Rac,↑Rho
$S1P_2$/EDG5	Widely distributed	$G_{i/0}$, $G_{q/12/13}$	↑↓AC,↑PLC,↑JNK,↑p38,↓Rac,↑Rho
$S1P_4$/EDG6	Lymphoid tissues	$G_{i/0}$	↑ERK,↑PLC
$S1P_5$/EDG8	Brain, spleen	$G_{i/0}$, G_{12}	↓AC,↓ERK,↑JNK,↑p54JNK

[a] For each receptor subtype, G-protein coupling, biological effects, and tissue distribution are indicated.

in a number of cell types, and often results in extracellular signal-regulated kinase (ERK) activation and inhibition of adenylyl cyclase [48,49]. Binding of S1P to $S1P_1$ activates phosphoinositide 3-kinase (PI3K) via G_i, leading to activation of the serine/threonine kinase Akt and phosphorylation of the Akt substrate, endothelial nitric oxide synthase (eNOS), shown to be involved in endothelial cell chemotaxis [50–53].

$S1P_1$ activation also regulates the activation state of small GTPases of the Rho family, namely Rac and Rho, which are downstream of the heterotrimeric G proteins and are involved in the regulation of cytoskeletal rearrangements [50,54,55]. $S1P_1$-induced G_i- and PI3K-dependent activation of Akt leads to the phosphorylation of $S1P_1$ at Thr^{236} located within the third intracellular loop [50]. This activates Rac and the subsequent signaling pathways required for cortical actin assembly, lamellipodia formation and chemotaxis [50]. In addition, in HEK 293 cells expressing $S1P_1$, S1P has also been shown to stimulate PTX-insensitive, $G_{12/13}$-mediated Rho pathways that regulate morphogenesis, such as adherens junction assembly and induction of placental (P)- and epithelial (E)-cadherin expression [4]. In contrast, $S1P_2$ and $S1P_3$, but not $S1P_1$, increase GTP-bound Rho in CHO cells [56]. Since $S1P_1$ cannot couple to $G_{12/13}$, it is therefore possible that Rho is activated through a different mechanism which has yet to be defined and which may also be dependent upon cell type. $S1P_1$ and $S1P_3$ were also found to regulate signaling pathways required for HUVEC morphogenesis into capillary-like networks [57]. Therefore, it is possible that $S1P_1$ activates Rho through cross-talk with $S1P_3$.

6. $S1P_2$ and $S1P_3$

Both $S1P_2$ and $S1P_3$ are widely expressed, with $S1P_3$ primarily expressed in the heart, lung, kidney and brain, whereas $S1P_2$ is abundant in the heart and lung, but less so in the brain of the adult rat and mouse [5,9,38,56]. However, $S1P_2$ is more prominent in the brain during embryonic development, suggesting a role for $S1P_2$-mediated signaling in neuronal development [58]. In contrast to $S1P_1$ null mice, $S1P_2$ and $S1P_3$ null mice were viable, fertile and developed normally [59,60], although deletion of both $S1P_2$ and $S1P_3$ resulted in marked perinatal lethality [60]. $S1P_2$ and $S1P_3$ couple to G_i, G_q, G_{12} and G_{13} [7,61,62]. Consequently, it has been demonstrated that $S1P_2$ and $S1P_3$ are coupled to the stimulation of phospholipase C (PLC) and Ca^{2+} mobilization via both PTX-sensitive and PTX-insensitive G proteins, most likely G_i and $G_{q/11}$, respectively [7,63–65]. Recently, it was also shown that PLC activation is significantly attenuated in $S1P_3$-null MEFs, yet is unaffected by deletion of $S1P_2$, suggesting that S1P-dependent PLC activation is preferentially mediated by $S1P_3$ [59,60]. $S1P_2$ and $S1P_3$ also regulate MAPK activation almost exclusively via G_i in CHO cells [56], while $S1P_2$ activates JNK and p38MAPK in a PTX-insensitive manner [63]. While $S1P_2$ activation increased adenylate cyclase activity in CHO cells [64], direct coupling of $S1P_2$ to G_s has not been detected [44].

Both $S1P_2$ and $S1P_3$ activate Rho by a $G_{12/13}$-dependent mechanism, resulting in stress fiber formation, cell rounding, neurite retraction and serum response element-driven transcriptional activation [9,56,66,67]. In contrast to PLC activation, S1P-induced Rho activation is unchanged in $S1P_3$-null MEFs but is significantly reduced by $S1P_2$ deletion, indicating that Rho activation is preferentially coupled to $S1P_2$ [59,60]. Interestingly, expression of $S1P_1$ or $S1P_3$ in CHO cells activates Rac in a PI3K-independent manner, while $S1P_2$ inhibits Rac activation and subsequent

membrane ruffling and cell migration [68]. These observations may be physiologically relevant as $S1P_2$ is expressed in cells in which S1P inhibits cell migration, such as melanoma and vascular smooth muscle cells [68].

7. $S1P_4$ and $S1P_5$

$S1P_4$ and $S1P_5$ are the most recently identified and therefore the least well characterized S1PRs. $S1P_4$ has a highly restricted expression pattern, being expressed primarily in lymphoid and hematopoeitic tissues, as well as in the lung [38,56]. $S1P_5$ is expressed in a variety of tissue types but is highly expressed in the white matter of the brain and in the spleen [38,56,69,70]. $S1P_4$ mediates S1P-induced PLC activation, intracellular Ca^{2+} mobilization, and MAPK activation, in a PTX-sensitive manner [38]. $S1P_5$ couples to $G_{i/0}$ and G_{12} but not to G_s or $G_{q/11}$ [69,71]. In CHO cells transfected with $S1P_5$, S1P-inhibited forskolin-induced cAMP accumulation was PTX-sensitive while activation of JNK and inhibition of serum-induced activation of ERK1/2 was PTX-insensitive [71]. The inhibitory effect of S1P on ERK1/2 activity was abolished by treatment with orthovanadate, suggesting the involvement of a tyrosine phosphatase [71].

8. S1PR signaling in angiogenesis

One of the most important biological roles of S1PRs is in angiogenesis, the process of new blood vessels formation from pre-existing ones. This process is an integral component of many physiological events, such as embryonic development, wound healing, and the menstrual cycle, each of which are defined by a requirement for new vessel formation to simultaneously supply oxygen and nutrients [72]. Angiogenesis is also critically important in a number of pathological conditions associated with blood vessel formation, including solid and hematologic tumor progression, chronic inflammation in rheumatoid arthritis and Crohn's disease, endometriosis, and diabetic retinopathy [72]. The process of angiogenesis involves a number of steps; 1) initiation; 2) endothelial cell migration and proliferation; 3) differentiation; and 4) maturation of the neovasculature. Recent studies have suggested that these steps are regulated by S1P-dependent activation of $S1P_1$ [47,50,55,57,73,74].

9. Initiation of angiogenesis

Vascular endothelial growth factor (VEGF), an important mediator of angiogenic initiation, is known to act on VEGF receptors (VEGFRs) to induce vasodilatation via NO production and increased endothelial cell permeability, allowing plasma proteins to enter the tissue and form a fibrin-rich provisional network. To date, there are three known VEGF tyrosine kinase receptors; VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1) and VEGFR-3 (Flt-4). VEGFR-1 and VEGFR-2 are expressed mainly in the vascular endothelium whereas VEGFR-3 is mostly restricted to the lymphatic endothelium [72]. Recent studies demonstrated that S1P activation of $S1P_1$ results in Akt-dependent phosphorylation of eNOS and increased NO [50,51,53,75]. This suggests that $S1P_1$ activation may affect vasodilatation in conjunction with VEGF.

10. Endothelial cell migration, proliferation and morphogenesis

Directional endothelial cell motility is driven by a number of chemoattractants that bind GPCRs (interleukin-8 and fMLP) or growth factors, such as VEGF and fibroblast growth factor (FGF) [72,76,77]. Several studies have shown that $S1P_1$ is a critical regulator of endothelial cell migration and proliferation [50,51,55,73,78–80]. However, inhibition of NO production had no effect on S1P-induced endothelial cell chemotaxis, whereas VEGF-dependent chemotaxis was blocked [51].

$S1P_1$ activation also regulates many of the components that are involved in morphogenesis. S1P stimulation of $S1P_1$ and $S1P_3$ expressed in HUVECs results in activation of $\alpha_v\beta_3$- and β_1-containing integrins [54]. In addition to regulating cell spreading and migration, antagonists of $\alpha_v\beta_3$ and β_1-containing integrins inhibited S1P-induced endothelial cell morphogenesis in a three-dimensional fibrin matrix [54]. Activation of $S1P_1$ and $S1P_3$ also activate Rac- and Rho-dependent adherens junction assembly and cytoskeletal rearrangement that ultimately result in differentiation into capillary-like networks [57]. Rac and Rho are involved in S1P-stimulated translocation of VE-cadherin and β-catenin to cell-cell junctions [57]. Interestingly, in contrast to the action of S1P, VEGF disrupts adherens junctions [36,57].

11. Maturation of neovasculature

Once the neovasculature has been formed, endothelial cells must deposit a new basement membrane and recruit surrounding vessel layers composed of mural cells, such as pericytes in small vessels and smooth muscle cells in large vessels [76,77,81]. Recruitment of mural cells is largely dependent upon the synthesis and secretion of PDGF within endothelial cells [72]. On endothelial cell-mural cell contact, a latent form of transforming growth factor-β (TGF-β), produced by both endothelium and mural cells, is activated in a plasmin-mediated process [76] and induces changes in myofibroblasts and pericytes, leading to the formation of a quiescent vessel, ECM production and maintenance of growth control [76].

Studies on $S1P_1$ knockout mice showed that it is essential for vascular maturation as its gene disruption resulted in impaired vascular maturation due to the failure of mural cells to migrate to arteries and capillaries to reinforce them [47]. In fact, although $S1P_1$ null embryos died in utero due to massive hemorrhage, they exhibited normal vasculogenesis and a substantially normal blood vessel network, yet were severely impaired in the recruitment of smooth muscle cells and pericytes to the vessel walls and this was attributed to their defective migration [47]. Extracellular S1P can directly stimulate $S1P_1$ on vascular smooth muscle cells (VSMCs), facilitating their migration to vessel walls or, alternatively, can stimulate $S1P_1$ expressed in endothelial cells that in turn may recruit VSMCs [47]. Recent studies have demonstrated that the effect of $S1P_1$ on vascular maturation can be attributed to the cross-talk between $S1P_1$ and PDGF receptor signaling [55,80] (Fig. 2). Cell migration toward PDGF, which stimulates SPHK and increases intracellular S1P, was dependent upon $S1P_1$ expression in a number of cell types, including HEK 293 cells, human aortic smooth muscle cells (ASMCs) and MEFs [55]. It was therefore suggested that spatially and temporally localized generation of S1P by activation of SPHK in response to PDGF results in restricted activation of $S1P_1$ that in turn activates Rac, resulting in an increase in cell

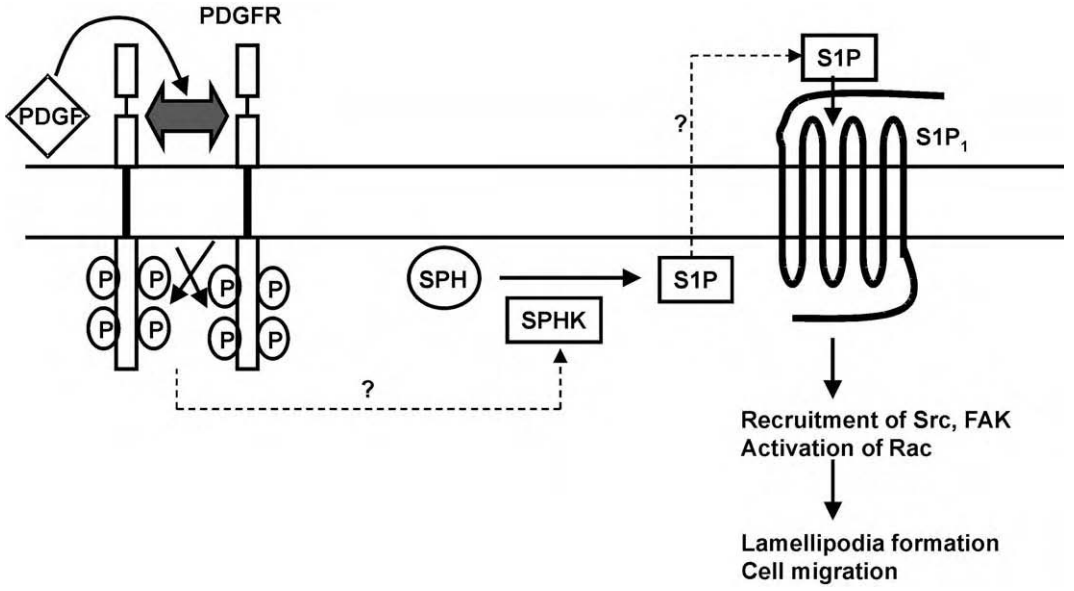

Fig. 2. Cross-talk between PDGFR and $S1P_1$ and its role in cell migration. Cell migration towards PDGF, which stimulates SPHK and increases intracellular S1P, has been shown to be dependent upon $S1P_1$ expression. PDGF-dependent-generation of S1P by activation of SPHK results in $S1P_1$-dependent activation of Rac, leading to cell migration towards PDGF.

motility [55]. Moreover, PDGF-induced cytoskeletal rearrangements, lamellipodia extensions and cell motility are abrogated in $S1P_1$ null fibroblasts [80]. Also, PDGF-induced focal adhesion formation and activation of FAK, Src and SAPK 2 were disregulated in the absence of $S1P_1$ [80]. However, $S1P_1$ was not involved in mitogenicity and survival effects induced by S1P or PDGF [80]. Hence, it was suggested that $S1P_1$ acted as an integrator linking the PDGFR to lamellipodia extension and cell migration [55,80].

12. The role of S1PR cross-talk in S1P signalling

As outlined above, many of the effects induced by extracellular S1P can be attributed to cross-talk between different receptors. For instance, activation of $S1P_1$ and $S1P_3$ is required for the activation of Rho and integrin in HUVECs, yet activation of Rac only requires $S1P_1$ [54,57]. Also, proliferation of human aortic endothelial cells requires both $S1P_1$ and $S1P_3$ signaling [73]. Cross-talk between $S1P_1$ and $S1P_2$ is also involved in the activation of ERK1/2 in C6 glioma cells [44]. Additionally, cross-talk has also been described between $S1P_1$ and the PDGFR, suggesting that further cross-talk mechanisms may exist between other receptor family members [55,80]. In fact, it has recently been demonstrated that $S1P_1$ can be phosphorylated in an agonist-independent manner *via* the activation of PKC [46] as well as in an agonist-dependent manner *via* GRK2 activation (Fig. 3). Hence, it is possible that $S1P_1$ may also be regulated by other receptor signaling mechanisms through PKC activation.

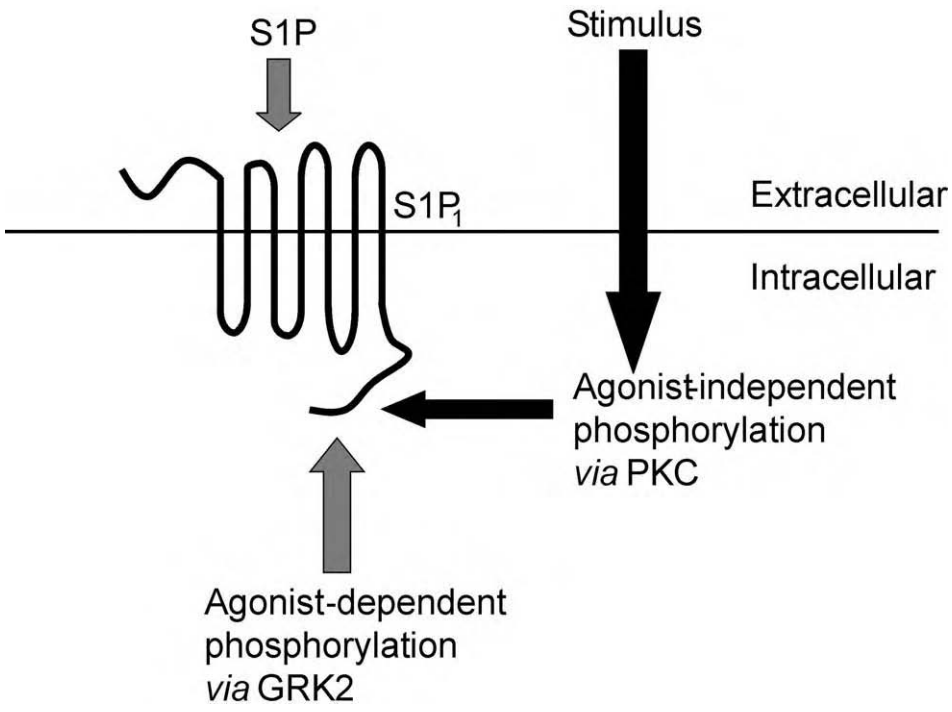

Fig. 3. Potential involvement of PKC in S1P-mediated phosphorylation of $S1P_1$. Binding of S1P to $S1P_1$ can lead to activation of phosphorylation of $S1P_1$. $S1P_1$ can also be phosphorylated in an agonist-independent manner *via* the activation of PKC. See text for more information.

The exact mechanisms involved in such cross-talk mechanisms constitute an interesting and rapidly growing aspect of S1PR research. One intriguing possibility is that receptor cross-talk is influenced by receptor dimerization. A recent study has demonstrated that a high degree of dimerization exists within the S1PR family [82]. For example, $S1P_1$, $S1P_2$ and $S1P_3$ have been shown to exist as monomers and as dimers independent of agonist activation [82]. Interestingly, dimerization has also been shown between $S1P_1$ and $S1P_3$, $S1P_1$ and $S1P_2$ and also $S1P_1$ and $S1P_2$ [82]. The implications of S1PR dimerization in terms of signaling are unknown. However, the possibility remains that many of the effects requiring more than one S1PR may be influenced by receptor dimerization. It is also possible that S1PRs may form complexes with other receptor subtypes. It was previously shown that co-stimulation of airway smooth muscle cells with S1P and PDGF elicits stronger p42/p44 MAPK activation than each agonist alone [83]. It was subsequently demonstrated that the PDGFR can also form a tethered complex with $S1P_1$ [84]. This complex enables the PDGFR to induce more efficient tyrosine phosphorylation of $G_{\alpha i}$ released upon stimulation of $S1P_1$ and that tyrosine phosphorylation of $G_{\alpha i}$ was required for PDGF and S1P to stimulate the p42/p44 MAPK pathway [84]. Interestingly, stimulation of the p42/p44 MAPK pathway promotes endothelial cell entry into the cell cycle, and induces transcription of VEGF, all of which are important in the process of angiogenesis [85].

13. Future directions and conclusions

Within the past few years there has been much progress in understanding the signaling properties and functions of the different S1PRs. Many studies point to an important role of $S1P_1$ in vascular maturation and angiogenesis . Much less is still known of the physiological and pathological functions of the other S1PRs. A better understanding of S1P signaling pathways, whether intra- or extracellular, should be useful in identifying targets for the development of therapeutics for a number of disease states. For example, there is much interest in the development of $S1P_1$ antagonists and/or SPHK inhibitors for the treatment of cancer since S1P plays such an important role regulating endothelial cell proliferation, survival, migration and vascularization, all critical processes in cancer progression. Development of specific S1PR agonists and antagonists should allow for a more accurate delineation of the effects of these receptors and provide potentially useful new therapeutics specifically targeting this novel sphingolipid metabolite.

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Appendix 6

Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways

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Abstract

S1P (sphingosine 1-phosphate) is the ligand for a family of specific G-protein-coupled receptors that regulate a wide variety of important cellular functions, including vascular maturation, angiogenesis, cell growth, survival, cytoskeletal rearrangements and cell motility. However, S1P also may have intracellular functions. In this review, we discuss two examples that clearly indicate that intracellularly generated and exogenous S1P can regulate biological processes by divergent pathways.

Introduction

S1P (sphingosine 1-phosphate), a sphingolipid metabolite found in organisms as diverse as plants, yeast, nematode worms, flies and mammals, is a potent regulator of a wide spectrum of important biological processes [1]. S1P is produced by phosphorylation of sphingosine catalysed by SphK (sphingosine kinase), a highly conserved enzyme that is activated by many agonists and stimuli [1,2]. S1P actions are mediated by binding to a family of five specific G-protein-coupled receptors, $S1P_1$–$S1P_5$, which are differentially expressed and coupled to a variety of G-proteins [1,3–5]. There is no doubt that the most important functions of S1P are mediated through its receptors, and these include regulation of angiogenesis, vascular maturation, cardiac development, neuronal survival and immunity [1,3,4]. The importance of these receptors has been most clearly shown by mutations and disruptions of S1P receptor genes. For example, mutations in $S1P_2$ lead to abnormal split heart development in the zebrafish [6]. Moreover, deletion of $S1P_1$ in mice revealed that it is essential for vascular maturation and migration of smooth muscle cells and pericytes around newly formed endothelium [7]. An exciting recent development with important clinical implications for S1P receptor signalling was the finding that the immunosuppressive drug FTY720, a sphingosine analogue, is phosphorylated by SphK to an active phosphorylated form which then, acting via S1P receptors, induces lymphopenia [8,9]. These studies implied a role for S1P receptors in lymphocyte homing and immunoregulation.

Other studies, however, suggested that S1P also has second messenger functions important for regulation of calcium homoeostasis [10,11], cell growth [12–14] and suppression of apoptosis [15–17]. Dissection of the intra- and extracellular actions of S1P is difficult, as intracellular targets have not been definitively identified and it has been shown that S1P, by binding to its receptors, can stimulate SphK to increase its own intracellular levels [18]. On the other hand, growth factors, such as PDGF (platelet-derived growth factor), can bind to their receptors to activate and recruit SphK to the leading edge of the cell [19], where it produces S1P to spatially and temporally stimulate cell-surface $S1P_1$ in an autocrine or paracrine manner [20] leading to activation of downstream signals crucial for cell movement [19,20]. It has been proposed that the PDGF receptor is in fact tethered to $S1P_1$, providing a platform for integrative signalling by these two types of receptor [21]. Another complicating factor in discriminating between intra- and extracellular actions of S1P is the fact that it can be specifically transported into cells by the cystic fibrosis transmembrane regulator (CFTR) [22], which could function to terminate extracellular signals as well as to initiate intracellular signals. In this review, we will describe two examples that show that exogenous and intracellularly generated S1P can affect cellular processes by distinct pathways.

SphK1 and generation of intracellular S1P, but not exogenous S1P, potentiate TNF-α (tumour necrosis factor α)-stimulated BH_4 (tetrahydrobiopterin) biosynthesis in C6 glioma cells

In astroglial cells, the biosynthesis of BH_4, the coenzyme required for NO synthesis and hydroxylation of tyrosine and tryptophan, is stimulated by various proinflammatory cytokines, including TNF-α. These induce expression of GTP cyclohydrolase, the rate-limiting enzyme in the *de novo* pathway for BH_4 biosynthesis. We observed that TNF-α stimulates iNOS (inducible nitric oxide synthase), which

Key words: cell growth and survival, G-protein-coupled receptor (GPCR), sphingosine kinase, sphingosine 1-phosphate (S1P), sphingosine 1-phosphate receptor, tetrahydrobiopterin.

Abbreviations used: BH_4, tetrahydrobiopterin; ERK, extracellular-signal-regulated kinase; S1P, sphingosine 1-phosphate; SphK, sphingosine kinase; PDGF, platelet-derived growth factor; TNF-α, tumour necrosis factor α; iNOS, inducible nitric oxide synthase; dihydro-S1P, sphinganine 1-phosphate.

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requires BH_4 for its activity, and GTP cyclohydrolase expression by divergent pathways [23]. Whereas TNF-α stimulated iNOS expression via a ceramide-dependent pathway, TNF-α regulated GTP cyclohydrolase expression independently of ceramide generation, and the sphingolipid metabolite S1P, but not ceramide, potentiated GTP cyclohydrolase mRNA expression induced by TNF-α. Conversely, TNF-α-induced SphK activation and generation of S1P did not modulate expression of iNOS. Thus TNF-α induces the co-ordinate expression of iNOS and GTP cyclohydrolase via ceramide- and S1P-dependent pathways, respectively [23]. Differential regulation of iNOS expression and levels of its cofactor BH_4 may be physiolologically relevant, as BH_4 has other important functions, especially in hydroxylation of the aromatic amino acids. Thus cytokine-dependent induction of iNOS and BH_4 synthesis should be co-ordinately regulated only in cell types where NO production is important.

As TNF-α stimulates GTP cyclohydrolase expression via a ceramide-independent pathway [23] and also increases S1P levels [24–26], it was of interest to investigate the role of SphK and S1P in the regulation of GTP cyclohydrolase and BH_4 biosynthesis. We found that stimulation of SphK and generation of intracellular S1P by TNF-α was involved in the regulation of GTP cyclohydrolase expression and activity, as the SphK inhibitor, *N*,*N*-dimethylsphingosine, completely blocked the potentiation effect of SphK on TNF-α-induced GTP cyclohydrolase and BH_4 synthesis [27]. Remarkably, exogenous S1P or dihydro-S1P (sphinganine 1-phosphate), which both bind to and activate all of the S1P receptors, did not mimic the effect of overexpression of SphK and increased intracellular S1P on GTP cyclohydrolase expression and activity or on BH_4 biosynthesis [27]. This was not due to a failure to activate S1P receptors on C6 cells, as binding of S1P and dihydro-S1P to $S1P_1$ present on C6 cells markedly activated ERK (extracellular-signal-regulated kinase). It was previously shown that activation of ERK by S1P leads to induction of expression of Egr-1, one of the immediate early gene products required for expression of this essential transcription factor for fibroblast growth factor-2, an autocrine factor that stimulates proliferation of astroglial cells [28,29]. Interestingly, overexpression of SphK1 not only potentiated TNF-α-induced BH_4 biosynthesis, but also, similar to its effect on fibroblasts [14,30], enhanced proliferation of C6 cells. However, whereas this proliferative effect could be blocked by PD90859, an inhibitor of the ERK pathway, similar treatment had no effect on TNF-α-induced BH_4 biosynthesis. Thus, although some effects of S1P, such as ERK activation, are clearly mediated through interactions with S1P receptors, BH_4 biosynthesis is only regulated by intracellularly generated S1P, not by exogenous S1P, and GTP cyclohydrolase may be an intracellular target of S1P.

One of the important functions of astroglial cells is to support neuronal cells by secreting a variety of neurotrophic factors, such as fibroblast growth factor-2 and other peptide growth factors, and neurotransmitters [31]. It should be noted that S1P can act in an autocrine or paracrine manner through different members of the S1P receptor family present on both astroglial and neuronal cells. S1P may therefore be another factor provided by glial cells *in vivo* to promote neuronal cell survival and morphology rearrangements and remodelling of the actin cytoskeleton during various stages of development. Alternatively, it could have pathophysiological effects at sites of brain lesions and alter the blood–brain barrier [32].

SphK promotes growth and survival independent of S1P receptors

Expression of SphK1 elevated intracellular levels of S1P, expedited the G_1/S transition, protected against apoptosis [14] and enhanced tumour formation in mice [30,33]. Heterotrimeric G-proteins couple cell-surface receptors to signals that regulate proliferation and survival, and asynchronous activation of Gα subunits can lead to oncogenic transformation [34]. Moreover, in some cases it is the Gβγ dimers that mediate proliferation via ERK1/2 activation and promote cell survival by activation of phosphoinositide 3-kinase [35]. The many studies implicating G-protein-coupled S1P receptors in the biological activities of S1P have overshadowed its intracellular roles, mainly due to the difficulty of dissociating signals that originate at the cell surface from those potentially originating inside cells. Pertussis toxin, which ADP-ribosylates and inactivates G_i proteins, has frequently been used to implicate a $G\alpha_i$-mediated pathway in proliferative and survival effects induced by exogenous S1P [13,36,37]. However, while pertussis toxin did not affect the proliferation and cytoprotective effects induced by SphK1 overexpression, these were completely blocked by a SphK inhibitor [14]. In agreement, inhibiting α_i and α_q, but not $\alpha_{12/13}$, drastically reduced proliferation and ERK1/2 activation induced by exogenous S1P. In sharp contrast, blocking signalling of the various Gα subunits and Gβγ dimers, the G-proteins that S1P receptors couple to and signal through, did not influence growth and survival promoted by SphK1 and intracellularly generated S1P [38]. Thus although the mitogenic effect of exogenous S1P appears to be mediated by binding to cell-surface S1P receptors, S1P formed by overexpression of SphK1 promoted growth and survival independently of these receptors. In further support of this conclusion, expression of SphK1 markedly stimulated growth of pertussis toxin-treated $S1P_2$/$S1P_3$ double-knockout mouse embryonic fibroblasts, which then have no functional S1P receptors [38]. In contrast with the strong mitogenic effect of SphK1 in pertussis toxin-treated $S1P_2$/$S1P_3$ double-knockout embryonic fibroblasts, no significant responses were observed with exogenous S1P [38]. These results suggest that S1P receptors are dispensable for the mitogenic effect of SphK1 but contribute to that of exogenous S1P. Moreover, pertussis toxin slightly reduced the protective effects of serum and high concentrations of S1P. In contrast, pertussis toxin did not decrease the strong cytoprotective effect of SphK1 overexpression in either wild-type or S1P receptor-null embryonic fibroblasts [38]. In summary, even in the absence of all S1P receptor signalling, SphK1 still markedly induced growth and survival. These data indicate that exogenous and intracellularly generated

S1P affect cell growth and survival by divergent pathways. Moreover, although intracellularly generated S1P can signal inside-out to regulate cytoskeletal rearrangements and cell movement, this is not the case for the regulation of cell growth and suppression of apoptosis, which is independent of S1P receptors.

Several other lines of evidence further support the concept of intracellular actions of S1P. First, dihydro-S1P, which is identical to S1P and only lacks the 4,5-*trans* double bond, binds to all of the S1P receptors, yet does not mimic the effects of S1P on cell survival [13,16,19,24]. Secondly, elevation of intracellular S1P by microinjection mobilizes calcium [11] and enhances proliferation and survival [13,16,39]. Thirdly, SphK1 and conversion of sphingosine into S1P mediates vascular endothelial growth factor-induced activation of Ras leading to activation of the ERK pathway and cell division by inhibiting the GTPase-activating protein, RasGAP, without the participation of S1P receptors [40]. Fourthly, levels of phosphorylated long-chain sphingoid bases regulate yeast environmental stress responses and survival although they do not have any S1P receptors [41–43], in a manner reminiscent of the function of S1P in eukaryotic cells. Accumulated sphingoid bases in yeast induce G_0/G_1 arrest, and the yeast SphKs, Lcb4 and Lcb5, remove the sphingoid block, allowing progression to S phase [43]. Similarly, PDGF-induced activation of CDK2, a cyclin-dependent kinase that promotes cell-cycle progression, was dependent on SphK [44]. This might be due to nuclear formation of S1P, as the time course for CDK2 activation is similar to that of the increase in nucleoplasmic SphK activity and translocation to the nuclear envelope induced by PDGF [45]. Finally, S1P regulates guard cell aperture in plants, which do not have S1P receptors, by direct effects on heterotrimeric Gα proteins. Recent studies with the tobacco plant *Arabidopsis thaliana* suggest that S1P links the drought hormone, abscisic acid, to regulation of stomatal aperture and guard cell ion channels [46]. Interestingly, S1P regulated stomatal apertures and guard cell inward K^+ channels and slow anion channels only in wild-type *Arabidopsis* cells, but not in cells with a knockout of GPA1, their only heterotrimeric G-protein α subunit gene [46]. These results suggest that S1P may be able to activate G-proteins directly, independently of cell-surface receptors. Whether S1P can also activate heterotrimeric G-proteins in eukaryotic cells in a similar fashion remains to be determined.

In conclusion, the concept of intracellular actions of S1P independent of S1P receptors and 'inside-out signalling' is not only important for understanding S1P functions, but may have therapeutic implications for development of SphK inhibitors for treatment of cancer [30] and also for prevention of radiation-induced premature ovarian failure and infertility [16,39].

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SPHINGOSINE-1-PHOSPHATE: AN ENIGMATIC SIGNALLING LIPID

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The evolutionarily conserved actions of the sphingolipid metabolite, sphingosine-1-phosphate (S1P), in yeast, plants and mammals have shown that it has important functions. In higher eukaryotes, S1P is the ligand for a family of five G-protein-coupled receptors. These S1P receptors are differentially expressed, coupled to various G proteins, and regulate angiogenesis, vascular maturation, cardiac development and immunity, and are important for directed cell movement.

Sphingosine-1-phosphate (S1P) is derived from sphingosine — the backbone of most sphingolipids — and it is now emerging as a vital lipid mediator. Sphingosine was named in 1884 after the Greek mythological creature, the Sphinx, because of its enigmatic nature[1]. More than a century later we are just beginning to unravel the riddle of S1P. S1P was originally considered to be formed merely as an intermediate in the detoxification of sphingosine, by its phosphorylation and subsequent degradation[2] (FIG. 1), but since the discovery that S1P regulates cell growth[3,4] and suppresses programmed cell death[5], there has been an explosion of important physiological and pathophysiological processes that are reported to be regulated by S1P in higher organisms. S1P and its homologous phosphorylated long-chain sphingoid bases have been detected in plants, worms, flies, slime mould and yeast, and they also regulate important biological responses even in these lower organisms, which further highlights the importance of S1P as a signalling molecule. The puzzle of how such a simple molecule can have such diverse roles has been resolved by the discovery that it belongs to a class of lipid mediators that function not only inside cells but also as ligands (agonists) for specific cell-surface receptors.

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S1P synthesis and degradation

As is the case for other signalling molecules, S1P levels in cells are low and tightly regulated in a spatial–temporal manner by the balance between its synthesis and degradation (FIG. 1). The activity of sphingosine kinase (SphK; BOX 1), which catalyses the ATP-dependent phosphorylation of sphingosine, is stimulated by many agonists, which indicates that this is a central regulating enzyme of S1P. The degradation of S1P is mediated by two different pathways: one is the reversible dephosphorylation back to sphingosine by specific S1P phosphatases (BOX 1); the second is the irreversible degradation by a pyridoxal phosphate-dependent S1P lyase (Spl) (BOX 1) to hexadecenal and phosphoethanolamine, which are subsequently reused for the biosynthesis of phosphatidylethanolamine (FIG. 1).

Sphingosine kinases. SphKs are a distinct and newly discovered class of lipid kinase that have five conserved domains and are evolutionarily conserved. They are expressed in humans, mice, yeast and plants, with homologues in worms and flies. Two mammalian isozymes, which are known as SphK1 and SphK2 (BOX 1), have been characterized[6]. These are located predominantly in the cytosol, although small amounts are associated with membranes. It is not yet clear whether these enzymes are present within organelles or whether they are loosely bound to cellular membranes, although in yeast, Lcb4 — the main enzyme that catalyses the formation of long-chain base phosphates — has been localized to the Golgi and late endosomes[7] and endoplasmic reticulum (ER)[8]. SphK1 and SphK2 have different kinetic properties, tissue distribution and temporal expression patterns during development, which indicates that they carry out distinct cellular functions and might be regulated differently. Their structural diversity and complex pattern of tissue

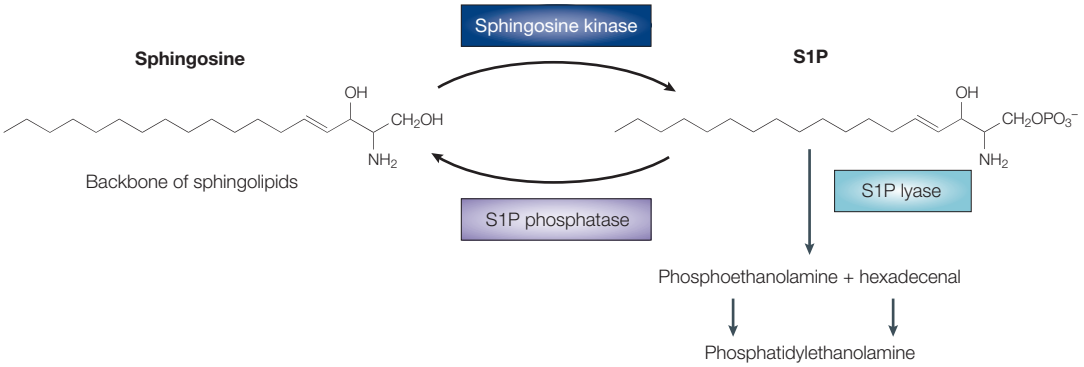


Figure 1 | **Formation and degradation of S1P.** The structures of sphingosine — the predominant sphingoid base of eukaryotic sphingolipids — and sphingosine-1-phosphate (S1P) are shown. Key metabolic enzymes for the formation and degradation of S1P are shown. Sphingosine kinases catalyse the formation of S1P from sphingosine. Conversely, two classes of enzyme — S1P phosphatases and S1P lyases — degrade S1P. The lyase products, hexadecenal and phosphoethanolamine, are reused for biosynthesis of phosphatidylethanolamine. See text and BOX 1 for further details.

expression is reminiscent of the large diacylglycerol kinase family, and it is probably not a coincidence that these two families share a high degree of homology in their catalytic domains. However, SphKs have a unique catalytic domain, which contains a consensus sequence of an ATP-binding site ($SGDGX_{(17–21)}K(R)$) that has some similarity to the highly conserved glycine-rich loop that is involved in binding the nucleotide in the catalytic site of many protein kinases[9].

S1P lyase. The main route of S1P degradation is through cleavage at the C2–C3 bond by Spl (BOX 1). The identification of the Spl gene in yeast[10], and subsequently in mammals and *Dictyostelium discoideum*, indicates that phosphorylated sphingoid base metabolism is a process that is conserved throughout evolution. Deletion studies have shown the importance of Spl and phosphorylated sphingoid bases in the regulation of global responses to nutrient deprivation in yeast[11]. Disruption of the *spl* gene in slime mould affects many stages of development, including the cytoskeletal architecture of aggregating cells, the ability to form migrating 'slugs' and terminal spore differentiation, which therefore implicates Spl in numerous processes in multicellular development[12]. It has been suggested that Spl is localized to the cytosolic face of the ER, at which all of the enzymes that are essential for sphingolipid biosynthesis reside (see below).

S1P phosphatase. Specific S1P phosphatases (SPPs) were first identified in yeast and shown to be important regulators of the heat-stress response[13–15]. On the basis of their homology with the yeast genes, the genes encoding two mammalian SPPs, SPP1 (REFS 16,17) and SPP2 (REF. 18), which only degrade phosphorylated sphingoid bases (BOX 1), were cloned. These SPPs belong to the family of magnesium-dependent, *N*-ethylmaleimide-insensitive type 2 lipid phosphate phosphohydrolases (LPPs)[19]. However, except for the conserved residues in three domains that are present in all LPPs[19], SPPs have little overall homology to other known LPPs. Moreover, whereas LPPs are present on the plasma membrane and function as so-called ecto-phosphatases to attenuate the actions of lysophosphatidic acid as an agonist of its cell-surface receptors[20], both SPP1 (REF. 21) and SPP2 (REF. 18), similar to their yeast counterparts[15], are localized to the ER[17,18].

Regulation of stress responses by S1P

S1P enhances growth and survival in diverse cell types[22]. By contrast, its precursors — ceramide (*N*-acyl sphingosine) and sphingosine — have generally been associated with growth arrest and cell death[23,24]. As these metabolites are interconvertible (FIG. 2), it has been proposed that it is not their absolute amounts, but rather their relative levels, that determine cell fate. The relevance of this 'sphingolipid rheostat' and its role in regulating cell fate has been borne out by the work of many researchers

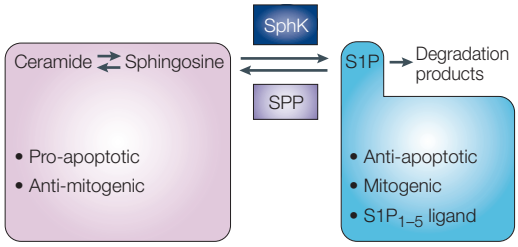


Figure 2 | **The sphingolipid rheostat: a conserved stress regulator.** Sphingosine-1-phosphate (S1P), sphingosine and ceramide are interconvertible sphingolipid metabolites. Many external stimuli have been shown to activate sphingosine kinases (SphKs) (BOX 3). SphKs converts sphingosine to S1P, thereby enhancing cell growth and survival. Conversely, S1P phosphatases (SPPs) remove the phosphate from S1P to form sphingosine, which is then *N*-acylated to form ceramide. Both ceramide and sphingosine have been associated with growth arrest and apoptosis. Whereas ceramide and sphingosine levels increase in response to many stress stimuli, suppression of apoptosis is associated with increases in S1P levels and decreases in ceramide (for reviews, see REFS 22,32,89). In addition, S1P regulates many processes by being able to interact with five specific cell-surface receptors (FIG. 4).

MITOCHONDRIAL OR INTRINSIC DEATH PATHWAY
The pathway that leads to death through the release, by stress-induced signals, of cytochrome *c* and other apoptogenic factors from the mitochondrial intermembrane space.

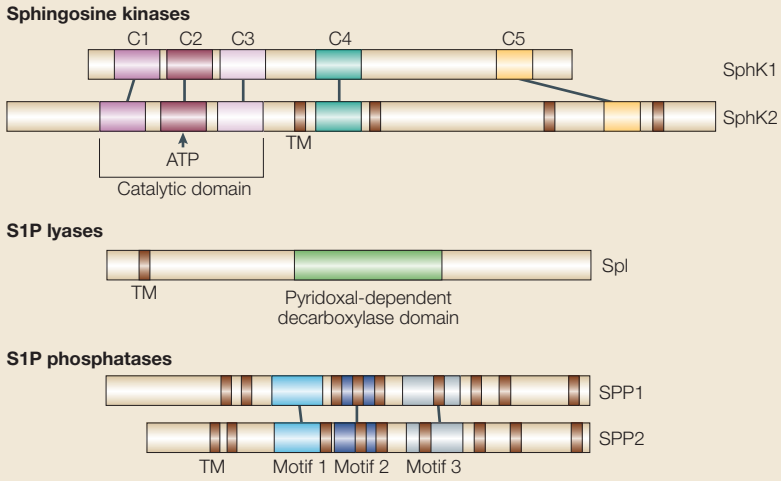
SPHINGOMYELINASE
An enzyme that catalyses the hydrolysis of sphingomyelin to ceramide (*N*-acylsphingosine) and choline phosphate.

CASPASES
A family of intracellular cysteine endopeptidases that have a crucial role in inflammation and mammalian apoptosis. They cleave proteins at specific aspartic acid residues.

using many different cell types and experimental manipulations.

A central finding of these studies is that SphKs and SPPs are essential regulators of the sphingolipid rheostat, and have converse effects on the pro-growth, anti-apoptotic messenger S1P versus the pro-apoptotic ceramide and sphingosine, and as a consequence, they have opposing effects on cell fate. For example, SphK1 and increased S1P levels enhance proliferation, expedite the G_1–S transition of the cell cycle and increase DNA synthesis[25]. SphK1 also promotes growth of cells in soft agar and tumour formation in mice, probably owing to its role in Ras and extracellular-signal regulated kinase (ERK)1/2 signalling[26-28]. In addition, SphK1 protects against apoptosis by inhibiting the MITOCHONDRIAL OR INTRINSIC DEATH PATHWAY, blocking the stress-activated protein kinase, Jun amino-terminal kinase (JNK)[25,29], and by activating nuclear factor κB (NF-κB)[30]. Deletion of both yeast SphK genes, *LCB4* and *LCB5*, showed that sphingosine is crucial for heat-induced G_0–G_1 arrest and that SphK removes the sphingosine block, allowing progression to S phase[31]. This indicates that there is a mechanism for cell-cycle control by the sphingolipid rheostat, which might be conserved in higher eukaryotes. Given the role of the sphingolipid rheostat in regulating growth and apoptosis, it is not surprising that sphingolipid metabolism is often found to be dysregulated in cancer, a disease that is characterized by enhanced cell growth, diminished cell death, or both.

Box 1 | Enzyme families that are important for regulating S1P levels



Sphingosine kinases

Two isoforms of sphingosine kinase (SphK) — Lcb4 and Lcb5 — were first cloned from *Saccharomyces cerevisiae* and show substantial sequence homology to mammalian SphK1 and SphK2 (REFS 80,81). SphK1 and SphK2 have five conserved domains (C1–C5) with the unique catalytic domain contained within C1–C3. The ATP-binding site ($SGDGX_{(17–21)}K(R)$) is present within C2. Hydropathy-plot analysis of the predicted amino-acid sequence of SphK1 does not indicate that any hydrophobic transmembrane regions (TM) are present, whereas SphK2 has four predicted transmembrane regions. Despite the overall homology between SphK1 and SphK2, the sequences of these two proteins diverge sufficiently to indicate that they might not have arisen from a simple gene-duplication event. Northern-blot analysis has shown that SphKs have different tissue distributions[6]: SphK1 expression is highest in lung and spleen, whereas SphK2 is most abundant in liver and heart. SphK1 expression is greatest at mouse embryonic day 7, whereas SphK2 expression is only detectable at embryonic day 11 and increases thereafter. Human SphK1 and SphK2 genes are on chromosomes 17q25.2 and 19q13.2, respectively.

S1P lyases

The S1P lyase (Spl) gene *BST1* (for bestowed of sphingosine tolerance) was first cloned from *S. cerevisiae*: mouse[82] and human[83] homologues were subsequently identified by sequence homology. The gene encoding human Spl is on chromosome 10q21 and is expressed most abundantly in the liver. Spl has one TM near its amino terminus and a conserved pyridoxal-dependent decarboxylase domain that contains several essential cysteine residues[83].

S1P phosphatases

Two genes that encode specific sphingoid base phosphate phosphatases (SPPs) have been identified in *S. cerevisiae* — *LBP1/YSR2/LCB3* and *LBP2/YSR3* (REFS 13,14,84). There are two mammalian SPPs, SPP1 (REF. 16) and SPP2 (REF. 18), which contain 8–10 predicted TMs and are highly specific for sphingoid base 1-phosphates. All SPPs contain three conserved motifs that are similar, yet distinct, to those in the lipid phosphate phosphohydrolase superfamily: motif 1, $KDX_{(4)}PRP$; motif 2, $EYX_{(2)}PSXH$; and motif 3, $LVX_{(3)}RXYXGMHX_{(2)}LD$. Although both mammalian SPPs have similar enzymatic properties and localize to the endoplasmic reticulum, SPP1 expression is highest in placenta and kidney, whereas SPP2 is most abundant in brain, heart, colon, kidney, small intestine and lung. The genes that encode human SPP1 and SPP2 are on chromosomes 14q23.1 and 2q36.3, respectively.

S1P and cell fate decisions

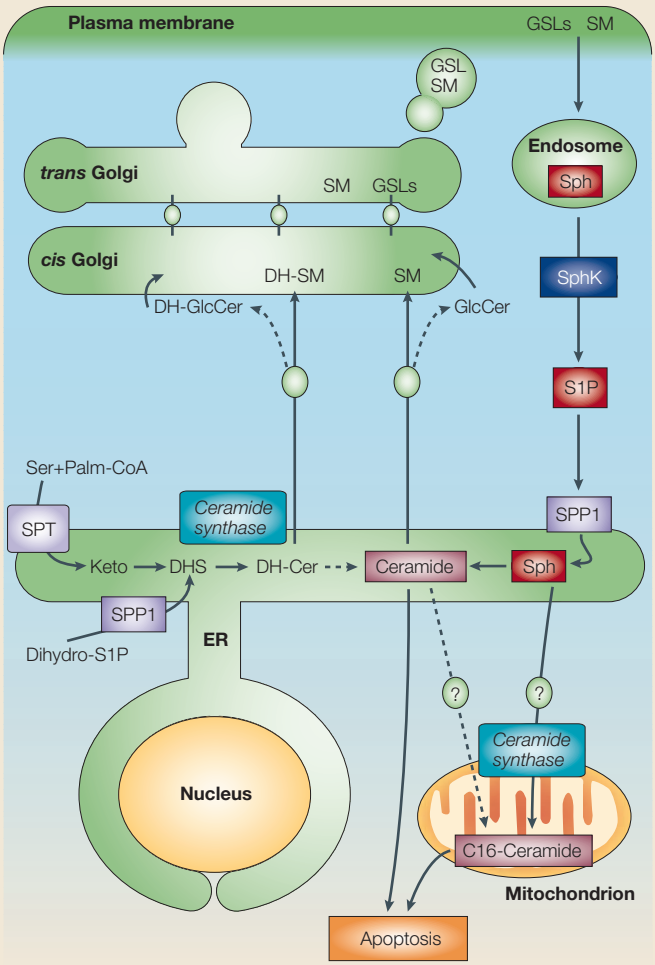
It was originally proposed that apoptotic ceramide was generated by the activation of one or more SPHINGOMYELINASES. Recent studies have also implicated ceramide that is generated from *de novo* sphingolipid biosynthesis[32-34] — predominantly *N*-palmitoylsphingosine (C16-ceramide) — in mitochondrial damage that leads to downstream activation of CASPASES and apoptosis[33]. There is convincing evidence that ceramide synthase and another ceramide biosynthetic enzyme, serine palmitoyltransferase, are activated during apoptosis[33-35] (BOX 2). However, because the ER contains other enzymes that are involved in ceramide biosynthesis as well as SPP1 and SPP2, and SPPs convert S1P to sphingosine (which can be further metabolized to ceramide), this indicates that SPPs could also have an important role in the regulation of ceramide biosynthesis. Indeed, expression of SPP1 altered the dynamic balance between S1P and sphingosine/ceramide in mammalian cells and, consequently, markedly enhanced apoptosis[16,21]. Likewise, the balance between the levels of ceramide and S1P, which is regulated by Lbp1, the yeast ER sphingoid base phosphate phosphatase, is crucial for survival and resistance to environmental stress in yeast[13-15]. This supports the idea that the sphingolipid rheostat is an evolutionarily conserved stress regulatory mechanism.

Ceramide versus dihydroceramide. Interestingly, whereas S1P could induce ceramide biosynthesis (particularly C16-ceramide) and apoptosis in *SPP1* transfectants, dihydro-S1P (which is also a substrate for SPP1 and SPP2) had no effect on ceramide levels, nor did it induce apoptosis[21]. In this regard, several inferences can

Box 2 | **The topology of sphingolipid metabolism and implications for apoptosis**

The *de novo* synthesis of all sphingolipids begins at the cytoplasmic face of the endoplasmic reticulum (ER) — which is where the enzymes that are required for ceramide biosynthesis are located — by the condensation of L-serine (Ser) with palmitoyl CoA (Palm-CoA), a reaction that is catalysed by serine palmitoyltransferase (SPT)[85,86]. In two rapid reactions, the product, 3-ketosphinganine (Keto), is reduced to sphinganine (dihydrosphingosine; DHS) and subsequently acylated by ceramide synthase to form dihydroceramide (DH-Cer). Dihydroceramide is then converted to ceramide by a desaturase[87]. Dihydroceramide and/or ceramide is translocated from the ER to the Golgi apparatus and then converted to sphingomyelin (SM) and dihydrosphingomyelin (DH-SM) by sphingomyelin synthase on the lumenal side of the Golgi, or to glucosylceramide (GlcCer) and dihydroglucosylceramide (DH-GlcCer) on the cytosolic surface of the Golgi[88]. After translocation into the Golgi lumen, glucosylceramide is further converted to lactosylceramide and more complex glycosphingolipids (GSLs). Sphingosine (Sph) produced from degradation of plasma membrane GSLs and SM in the endocytic (Endosome) recycling pathway might be used for formation of ceramide by sequential phosphorylation by sphingosine-1-phosphate (S1P) kinase (SphK) and dephosphorylation by S1P phosphatases (SPPs), or converted into pro-apoptotic C16-ceramide by ceramide synthase in the mitochondria.

The insertion of the *trans* 4,5 double bond into ceramide by the desaturase is an important step because ceramide, and not dihydroceramide, induces apoptosis[32]. Remarkably, although dihydro-S1P is also a substrate for SPP1 — it forms dihydrosphingosine, which is then converted to dihydroceramide by ceramide synthase — it does not cause significant ceramide accumulation or increase apoptosis in *SPP1* transfectants, in contrast to S1P[21]. This indicates that dihydroceramide might be more efficiently used for sphingomyelin and/or glycosphingolipid biosynthesis than ceramide is. Alternatively, the translocation of dihydroceramide to the Golgi might be more rapid than that of ceramide. So, the biosynthetic trafficking of ceramide and dihydroceramide might be different, and vesicular and non-vesicular transport pathways of ceramide versus dihydroceramide might carry out special functions. The figure is modified with permission from REF. 21 © the Rockefeller University Press (2002).



be made: first, SPP1 functions in an unprecedented manner to regulate the biosynthesis of ceramide, which has an essential role in apoptosis; second, the presence or absence of the *trans* double bond in the sphingoid bases dictates their function in the biosynthesis of ceramide; and third, ceramide and dihydroceramide could have different biosynthetic trafficking pathways (BOX 2) or, alternatively, vesicular and non-vesicular pathways of ceramide versus dihydroceramide transport might carry out special functions. As sites of contact have often been observed between the membranes of the ER and mitochondria[36], it is also possible that these could be responsible for delivery of apoptotic ceramide, but not dihydroceramide, to the mitochondria. As dihydroceramides are much less potent than ceramides in the induction of apoptosis, divergence in the regulation of ceramide and dihydroceramide synthesis has important implications, not only for sphingolipid metabolism, but also for their distinct roles in apoptosis. Sphingosine, but not sphinganine (also known as dihydrosphingosine, which has exactly the same structure as sphingosine but lacks the 4,5-*trans* double bond), formed at the ER by SPP1, might be transported to the mitochondria and function as a substrate for ceramide synthase, or for a new mitochondrial ceramidase with reciprocal (ceramide synthase) activity that shows a strong preference for sphingosine over dihydrosphingosine as substrate[37]. This ceramide could induce apoptosis that arises from mitochondrial damage, generation of reactive oxygen species, release of cytochrome *c* and subsequent activation of caspase activity.

S1P regulates ceramide biosynthesis and apoptosis. Although dihydrosphingosine is an intermediate in the biosynthesis of ceramide, it is not converted directly to sphingosine. Sphingosine arises predominantly by the turnover of membrane sphingolipids through the endocytic recycling pathway. Most of the sphingomyelins and glycosphingolipids that are present in slowly dividing cells are in fact made from this recycled sphingosine[38]. In yeast, deletion or mutation of the sphingosine kinase genes, *LCB4* and *LCB5*, or the ER sphingoid base phosphate phosphatase gene, *LBP1*, completely blocked the incorporation of added dihydrosphingosine into sphingolipids[13,14,39]. So, both SphK and SPP are required in this cycle of phosphorylation, uptake into the ER and dephosphorylation for the synthesis of sphingolipids through the endocytic pathway. Likewise, we suggest

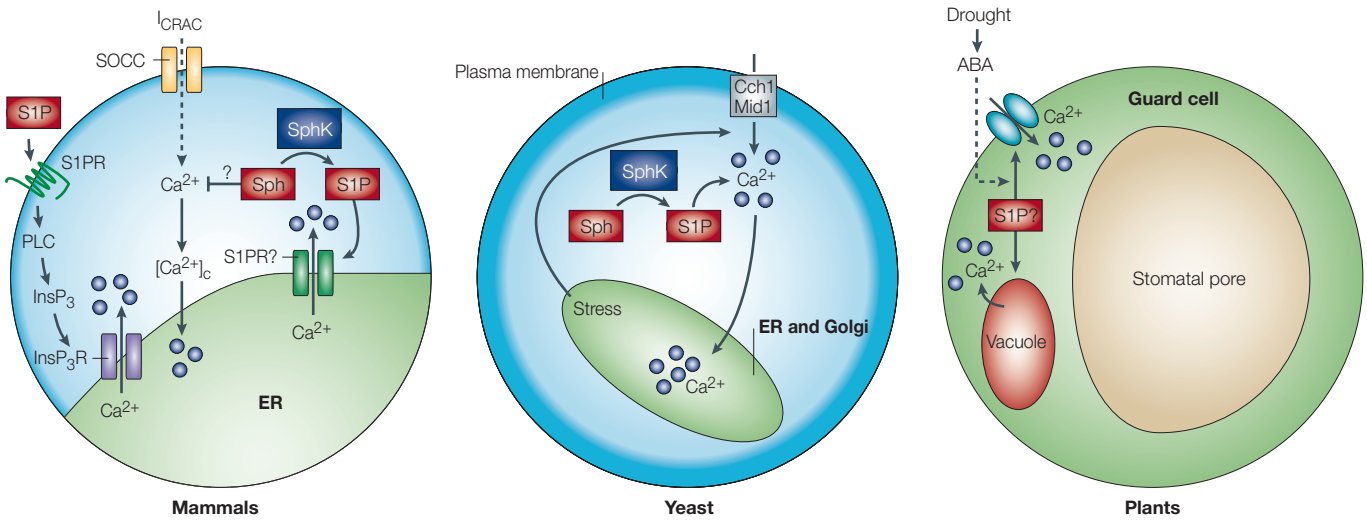


Figure 3 | **S1P regulates calcium homeostasis in plants, yeast and mammals. a** | In mammalian cells, it has been proposed that sphingosine kinase (SphK) converts sphingosine (Sph) — which inhibits the store-operated calcium release-activated calcium current (SOCC) I_{CRAC} — to sphingosine-1-phosphate (S1P), which mobilizes calcium independently of inositol trisphosphate ($InsP_3$) and its receptor ($InsP_3R$). However, the targets (S1P receptors; S1PRs) of S1P on the endoplasmic reticulum (ER) have not yet been identified, as denoted by the question mark. In addition, some S1P receptors on the plasma membrane are coupled to the activation of phospholipase C (PLC), formation of $InsP_3$ and calcium mobilization ($\uparrow[Ca^{2+}]_c$) (FIG. 4). **b** | In yeast, calcium influx in response to stress in the ER is mediated by calcium channels that are composed of Cch1, a homologue of the catalytic subunit of voltage-gated calcium channels, and a regulatory subunit, Mid1. The formation of S1P, which is catalysed by the SphKs Lcb4 or Lcb5, increases calcium influx to replenish secretory organelles with calcium. **c** | In plants, S1P is involved in the signal-transduction pathway that links the drought hormone abscisic acid (ABA) to the release of calcium from intracellular stores, notably the vacuole. It is still unclear how ABA regulates S1P levels and how S1P affects calcium.

that in mammalian cells, sphingosine is formed in late endosomes and lysosomes, then phosphorylated on their cytosolic surfaces by SphK (BOX 2), and the resulting S1P, owing to its more hydrophilic nature, is readily transported to the ER where it is dephosphorylated by SPP1 and/or SPP2 to sphingosine. This sphingosine is subsequently converted to ceramide, predominantly to the C16-ceramide species[16,21], by ceramide synthase. As the overexpression of SPP1, which dephosphorylates S1P, increases *de novo* synthesis of ceramide, it is possible that this increase could result from relief of the negative regulation of serine palmitoyltransferase or ceramide synthase by S1P. One piece of evidence in support of such a hypothesis is that the suppression of sphingoid base synthesis and downregulation of serine palmitoyltransferase by free sphingoid bases requires the conversion of these free bases to sphingoid base-1-phosphates[40]. It is intriguing to speculate that negative regulation by S1P of one of the key enzymes in the *de novo* ceramide biosynthesis pathway might be the mechanism for the pro-survival activity of S1P and its ability to suppress ceramide-mediated apoptosis, and that it might also explain why the relative levels of these interconvertible sphingolipid metabolites are tightly regulated.

PHOSPHOLIPASE C
A phosphoric diester hydrolase that splits the bond between the phosphorus atom and the oxygen atom at C1 of the glycerol moiety of a glycerophospholipid.

S1P and calcium homeostasis

From mammals… More than a decade ago, it was suggested that a sphingosine derivative formed in the ER[41], which was later identified as S1P[42,43], could mobilize calcium from internal sources in an inositol trisphosphate ($InsP_3$)-independent manner. Over the ensuing years, many external stimuli — beginning with crosslinking of the immunoglobulin receptor[44] — that were known to regulate calcium homeostasis without activation of PHOSPHOLIPASE C (PLC), have been shown to stimulate SphK[45,46]. Specific SphK inhibitors block the increases in intracellular calcium concentration that are elicited by these agents, which indicates the importance of SphK activity and, by inference, the generation of S1P (FIG. 3). In addition, the store-operated calcium release-activated calcium current (I_{CRAC}), which is important for replenishing calcium stores, is blocked by sphingosine. So, conversion to S1P, which is catalysed by SphK, would lower sphingosine levels leading to the disinhibition of I_{CRAC}, which would thereby also increase calcium influx[47]. Although at present there are several reports that support the idea that SphK is involved in calcium mobilization, this has become the subject of controversy, because intracellular targets in the ER that are responsible for the effects of S1P have still not been identified. Furthermore, the presence of S1P receptors that are coupled to PLC and calcium mobilization complicates this issue (discussed below). However, it should be remembered that the first studies on the mobilization of calcium in ER preparations by sphingosine showed that it required conversion of sphingosine to S1P, and that S1P itself mobilized calcium in these microsomal membranes in the absence of plasma membrane S1P receptors[43,48,49]. Moreover, S1P has recently been shown to be involved in regulating calcium levels in yeast and higher plants, which do not

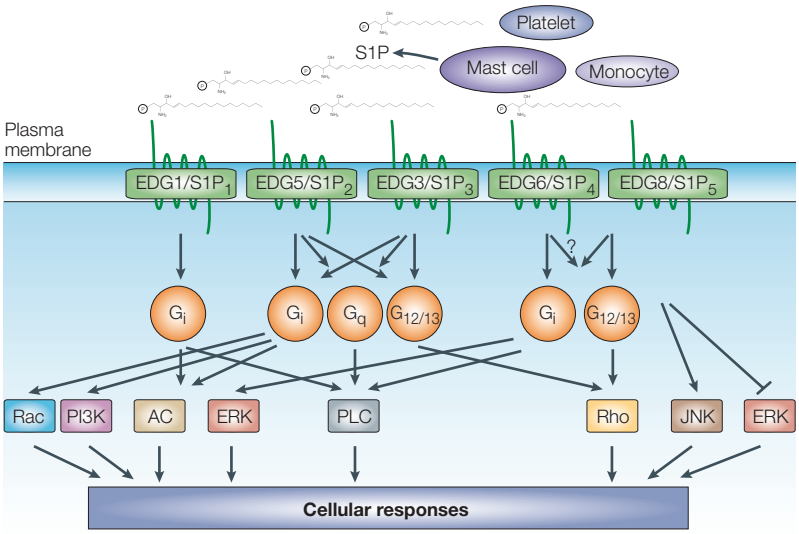


Figure 4 | **S1P is a ligand for five G-protein-coupled receptors.** Sphingosine-1-phosphate (S1P) in serum, which is secreted by mast cells, platelets and monocytes, binds to specific members of the S1P receptor family, which are coupled to different G proteins (for example, EDG1/$S1P_1$ and EDG6/$S1P_4$ couple mainly to G_i; both EDG5/$S1P_2$ and EDG3/$S1P_3$ activate G_i, G_q and $G_{12/13}$; and EDG8/$S1P_5$ is linked to G_i and $G_{12/13}$) leading to activation or inhibition of the indicated downstream signalling pathways. Only a few examples of these pathways are illustrated — in particular, extracellular signal-regulated kinase, ERK; Jun amino terminal kinase, JNK; the small GTPases of the Rho family (Rho and Rac); phospholipase C, PLC; adenylyl cyclase–cyclic AMP, AC; and phosphatidylinositol 3-kinase, PI3K. The reader is referred to recent reviews on signalling downstream of S1P receptors and the G proteins that they are coupled to[22,66].

have S1P receptors, and this indicates that S1P might have a universal intracellular signalling role in calcium regulation (FIG. 3a).

…to yeast and plants. Recent evidence indicates that yeast might use I_{CRAC}-like mechanisms to help replenish secretory organelles with calcium[50]. Calcium influx in response to stress in the ER (which can be induced by perturbations of protein, carbohydrate or lipid biosynthesis in the ER) is mediated by calcium channels that are similar to voltage-gated calcium channels. This calcium influx requires SphKs (Lcb4 and Lcb5), and is inhibited by Spl (Dpl1) and the SPP Lcb3 (REF. 50; FIG. 3b).

In higher plants, STOMATA form pores on leaf surfaces for the exchange of water and gases between the plant and the atmosphere. The plant hormone abscisic acid (ABA), the levels of which increase in response to drought, regulates stomatal pore size by mediating turgor changes in the GUARD CELLS that surround the pore. An increase in the cytosolic concentration of free Ca^{2+} is a common intermediate in many of the pathways that lead to either opening or closure of the stomatal pore. Recently, it was shown that S1P, but not dihydro-S1P, stimulates stomatal closure and Ca^{2+} mobilization in *Commelina communis* guard cells. In addition, the promotion of stomatal closure by ABA was attenuated by a SphK inhibitor, and finally, drought also increased S1P levels[51,52]. These results indicate that S1P is also important in calcium regulation and the control of guard-cell aperture in plants (FIG. 3c). So, it is intriguing to note that S1P regulates calcium both in yeast and plants, particularly in response to harsh environmental conditions.

STOMATA
The pores in the epidermis of plants, in particular in the leaves, through which gaseous exchange occurs.

GUARD CELLS
Cells that are found on the underside of plant leaves, which pair up to form stomata, or leaf pores. Guard cells control the size of the stomata, and so, in turn, regulate gas exchange in the leaf.

G-PROTEIN-COUPLED RECEPTOR
A seven-helix transmembrane-spanning cell-surface receptor that signals through heterotrimeric GTP-binding and -hydrolysing G-proteins to stimulate or inhibit the activity of a downstream enzyme.

HETEROTRIMERIC G PROTEIN
A component of receptor-mediated activation or inhibition of adenylyl cyclase and other second messenger systems.

S1P as an agonist of S1P receptors

Since the first report that S1P was the ligand for the orphan G-PROTEIN-COUPLED RECEPTOR (GPCR) endothelial differentiation gene 1 (EDG1)[53], it has become clear that the most important biological role of S1P is to function as the natural ligand for what has grown into the EDG family of GPCRs, which are now also known as S1P receptors. So far, five members — EDG1/$S1P_1$, EDG5/$S1P_2$, EDG3/$S1P_3$, EDG6/$S1P_4$, and EDG8/$S1P_5$ (REF. 54) — which bind only S1P and dihydro-S1P with high affinity, have been identified. They are ubiquitously expressed and couple to various G proteins that regulate numerous downstream signals (FIG. 4). This endows S1P with the ability to regulate diverse physiological processes, including angiogenesis and vascular maturation[55-58], heart development[59] and immunity[60-62] in a highly specific manner, depending on the relative expression of S1P receptors and G proteins. S1P receptors also differentially regulate the small GTPases of the Rho family, particularly Rho and Rac[56], which are downstream of the HETEROTRIMERIC G PROTEINS and are important for cytoskeletal rearrangements[63] and directed cell movement[64,65]. For example, $S1P_1$ regulates Rac-coupled cortical actin formation[56], and $S1P_2$ and $S1P_3$ activate Rho[65]. $S1P_2$ also blocks Rac activation[65], thereby inhibiting cell movement. These aspects have recently been covered in several comprehensive reviews[22,66,67] and are not discussed at length here. Rather, we focus our attention on a few examples that illustrate the roles of S1P receptors in development, as well as highlighting some recent advances.

S1P in cell migration: heart development. Coordinated cell migration is central to many physiological processes, including embryonic development, organogenesis, wound healing and the immune response. Recent studies indicate that S1P receptors have key roles in these processes. During development, cardiac precursor cells migrate from two patches of tissue to the dorsal midline and merge to form the heart. A mutation in the gene encoding the zebrafish homologue of $S1P_2$ causes 'miles apart' split-heart development (FIG. 5a). This was the first evidence that S1P has a role in cell migration during embryogenesis. Interestingly, the zebrafish Miles-apart protein does not need to be expressed on the migrating heart precursor cells themselves; rather, it is also expressed in PARAXIAL CELLS, which are located on either side of the midline. So, $S1P_2$ signalling might somehow enable these cells to direct cardiac precursor cells to the midline. Surprisingly, however, disruption of *$S1P_2$* in mice did not result in a similar phenotype[68].

S1P in cell migration: vascular development. An essential role for $S1P_1$ in vascular development and maturation was shown by gene disruption in mice. *$S1P_1$*-knockout mice died *in utero* between embryonic day 12.5 (E12.5) and E14.5 owing to vascular abnormalities that were caused by the defective migration of mural cells (vascular smooth muscle cells (VSMCs) and PERICYTES) around nascent blood vessels (FIG. 5b). Fibroblasts obtained from $S1P_1$-null embryos failed to

PARAXIAL CELLS
Cells of a region of the mesoderm, which is known as the paraxial mesoderm, that is adjacent to the notochord. The paraxial mesoderm becomes segmented rostrocaudally to give rise to the somites early in development.

PERICYTE
A support cell of the capillaries. Pericytes are known as smooth muscle cells in larger vessels.

IMMUNOMODULATOR
Any agent that alters the extent of the immune response to an antigen.

LYMPHOPENIA
A decrease in the number of lymphocytes in the blood, which might occur in various diseases.

show Rac activation and were unable to migrate towards S1P[57], indicating that S1P might function directly on $S1P_1$ that is expressed by VSMCs/pericytes to facilitate their recruitment and migration around the vessels. However, the indirect influence of the Miles-apart protein on migrating cells, together with the high level of expression of $S1P_1$ in endothelial cells, raises the possibility that S1P could also stimulate $S1P_1$ that is expressed on endothelial cells, and this in turn would recruit VSMCs[57].

What are the mechanisms by which cells expressing S1P receptors could regulate migration of another cell type? The activation of S1P receptors could upregulate adhesion molecules or cause other changes to the cell surfaces that would then be permissive for the recruitment of migrating cells. Or perhaps S1PR signalling could induce the release of components of the extracellular matrix or of a diffusible signal to attract migrating cells. Although much remains to be learned before this question can be answered definitively, there is evidence in the literature that supports either or both of these possible mechanisms. S1P has been shown to induce Rho-dependent activation of integrins[69], to affect vascular endothelial growth factor (VEGF) signalling[70] and to activate matrix-degrading proteinases[71].

S1P in lymphocyte migration. An unanticipated but important function for S1P receptors in lymphocyte migration and immune responses emerged from studies with the IMMUNOMODULATOR and sphingosine analogue FTY720, which is a drug that shows great potential for human kidney transplantation and the management of chronic autoimmune diseases such as multiple sclerosis. FTY720 elicits LYMPHOPENIA, which results from a reversible redistribution of lymphocytes from the circulation to secondary lymphoid tissues. Two recent reports show that FTY720 is phosphorylated by SphK1 and that the phosphorylated compound is a potent agonist of all S1P receptors except $S1P_2$ (REFS 60,61). Phosphorylated FTY720, which presumably functions through S1P signalling pathways, modulates chemotactic responses and lymphocyte trafficking to divert lymphocytes from inflammatory lesions and graft sites (FIG. 5c).

S1P receptors and receptor crosstalk

The importance of S1P in cell migration, angiogenesis[56], vascular maturation[57], vascular permeability[58] and tumour biology prompted a flurry of studies on its relationship with VEGF, one of the most important growth factors that is involved in the processes of

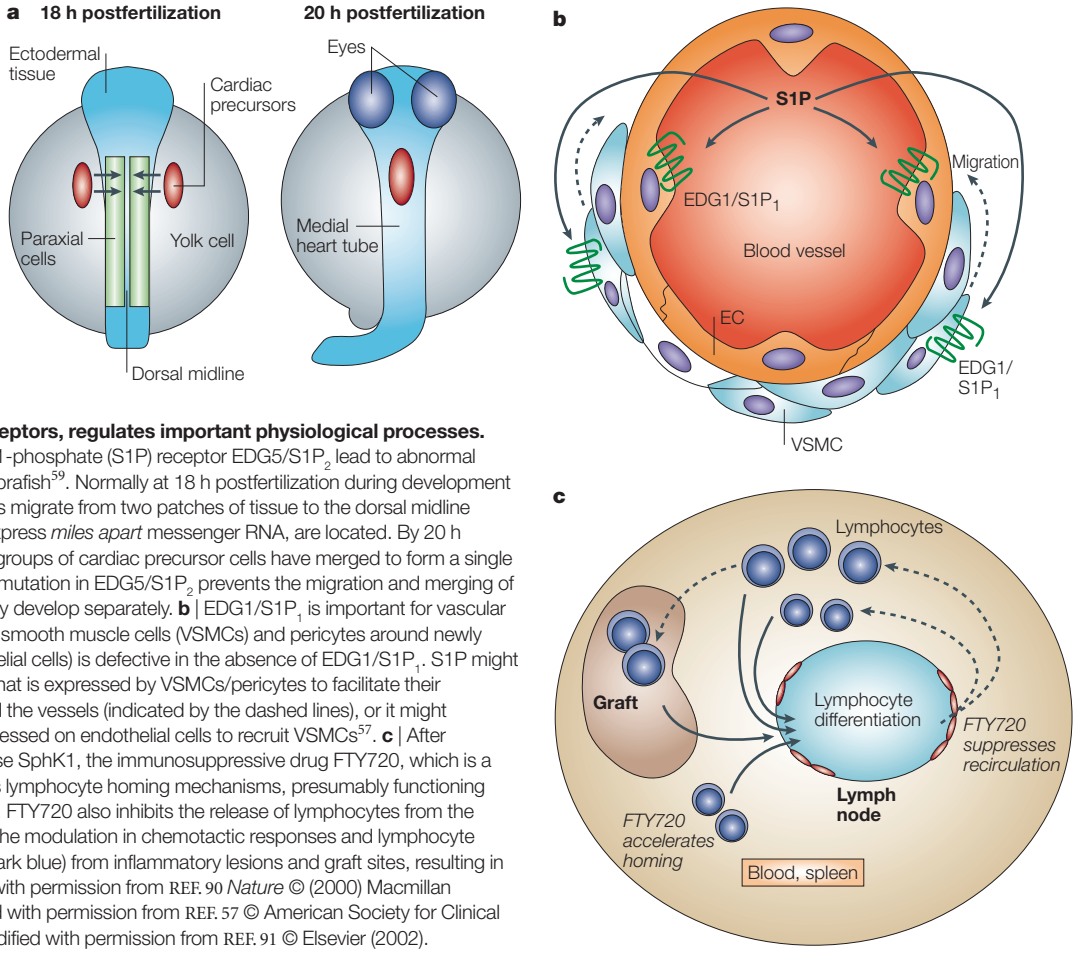


Figure 5 | **S1P, through S1P receptors, regulates important physiological processes.** **a** | Mutations in the sphingosine-1-phosphate (S1P) receptor EDG5/$S1P_2$ lead to abnormal split-heart development in the zebrafish[59]. Normally at 18 h postfertilization during development (left panel), cardiac precursor cells migrate from two patches of tissue to the dorsal midline where the paraxial cells, which express *miles apart* messenger RNA, are located. By 20 h postfertilization, the two bilateral groups of cardiac precursor cells have merged to form a single medial heart tube (right panel). A mutation in EDG5/$S1P_2$ prevents the migration and merging of these two groups of cells and they develop separately. **b** | EDG1/$S1P_1$ is important for vascular maturation. Migration of vascular smooth muscle cells (VSMCs) and pericytes around newly formed endothelium (EC, endothelial cells) is defective in the absence of EDG1/$S1P_1$. S1P might function directly on EDG1/$S1P_1$ that is expressed by VSMCs/pericytes to facilitate their recruitment and migration around the vessels (indicated by the dashed lines), or it might stimulate EDG1/$S1P_1$ that is expressed on endothelial cells to recruit VSMCs[57]. **c** | After phosphorylation by the S1P kinase SphK1, the immunosuppressive drug FTY720, which is a sphingosine analogue, stimulates lymphocyte homing mechanisms, presumably functioning through S1P signalling pathways. FTY720 also inhibits the release of lymphocytes from the lymph node into the circulation. The modulation in chemotactic responses and lymphocyte trafficking diverts lymphocytes (dark blue) from inflammatory lesions and graft sites, resulting in lymphopenia. Part **a** is modified with permission from REF. 90 *Nature* © (2000) Macmillan Magazines Ltd. Part **b** is modified with permission from REF. 57 © American Society for Clinical Investigation (2000). Part **c** is modified with permission from REF. 91 © Elsevier (2002).

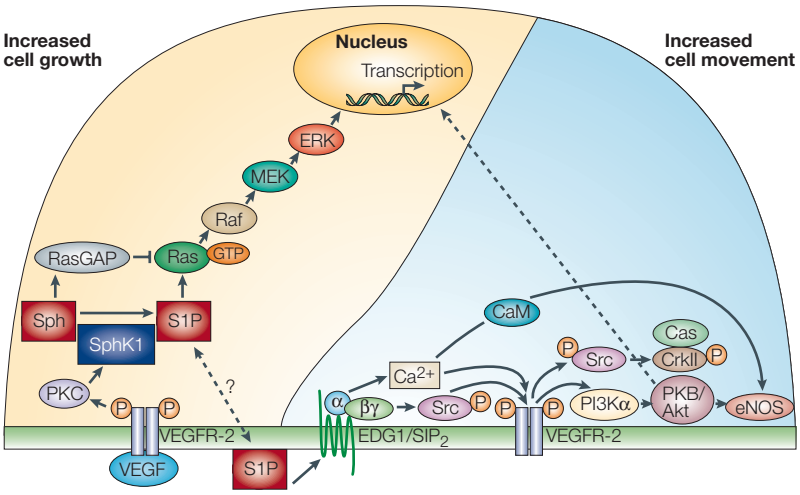


Figure 6 | **Crosstalk of VEGF and S1P signalling.** The crosstalk of sphingosine-1-phosphate (S1P) and vascular endothelial growth factor (VEGF) in cell movement is shown in the right-hand side of the figure (light-blue shading). S1P can activate VEGF receptor-2 (VEGFR-2) in the absence of added VEGF by receptor crosstalk. Ligation of the S1P receptor EDG1/$S1P_1$ and activation of G_i and $G_{\beta\gamma}$, in turn, lead to the activation of components such as Src that result in the phosphorylation of VEGFR-2. This transactivation of VEGFR-2 can then lead to activation of two signalling cascades that are important for movement and vascular remodelling: first, activation of Src-family tyrosine kinase(s) and the adaptor protein CrkII; and second, activation of phosphatidylinositol 3-kinase α (PI3Kα), protein kinase B (PKB)/Akt and endothelial nitric oxide synthase (eNOS), and the formation of nitric oxide (NO). The dashed arrow indicates that PKB/Akt is essential not only for cell migration but also for cell proliferation. In response to G_i-protein-mediated activation of phospholipase C, intracellular calcium (Ca^{2+}) levels increase. Ca^{2+} then complexes with calmodulin (CaM), which also activates eNOS. For simplicity, other known signalling pathways downstream of EDG1/$S1P_1$ (as indicated in FIG. 4) are not shown. The crosstalk of VEGF and S1P in cell growth is shown on the left-hand side of the figure (light yellow shading). After binding VEGF, VEGFR-2 activates protein kinase C (PKC), which then stimulates the S1P kinase SphK1. This increases S1P levels with concomitant decreases in sphingosine (Sph) and inhibition of the GTPase-activating protein, RasGAP[28]. Activation of Ras, in turn, will lead to activation of Raf, MAPK and ERK kinase (MEK), extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and, eventually, to DNA synthesis. The question mark and dashed arrow in this part of the figure indicate that it is still not known whether S1P that is generated intracellularly by VEGF can stimulate S1P receptors to regulate cell movement.

vasculogenesis and angiogenesis. These studies have shown that there is very complex crosstalk between these two molecules. S1P, similar to various agonists of GPCRs, can activate growth factor tyrosine kinase receptors in the absence of added growth factors, which indicates that receptor tyrosine kinases might be activated through receptor crosstalk (which is also known as transactivation).

Implications for angiogenesis. Two recent studies have shown that S1P can transactivate VEGF receptors in endothelial cells (FIG. 6). In the first study, activation of one of the VEGF receptors — VEGF receptor 2 (VEGFR-2/Flk-1) — and the subsequent activation of Src-family tyrosine kinases were required for S1P-induced phosphorylation of the adaptor protein CrkII in human umbilical vein endothelial cells (FIG. 6). In agreement with the established role of CrkII, S1P-promoted membrane ruffling and cell motility were also decreased by an inhibitor of VEGFR-2 (REF. 72). Similarly, S1P transactivated VEGFR-2 on endothelial cells through a signalling pathway that involved G_i, calcium and Src-family tyrosine kinases[70]. Unexpectedly, the transactivation of VEGFR-2 by S1P was independent of the release of endogenous VEGF, because a neutralizing antibody did not block the effect of S1P, in contrast to Src inhibitors. Taken together with other studies, it seems that activation of $S1P_1$ leads to G_i-dependent activation of the β isoform of phosphatidylinositol 3-kinase (PI3Kβ) and PLC. This results in an increased concentration of intracellular calcium, which complexes with calmodulin to activate endothelial nitric oxide synthase (eNOS)[73]. eNOS produces nitric oxide (NO), which has a crucial role in the regulation of vascular tone, vascular remodelling and VEGF-induced angiogenesis. Simultaneously, transactivation of VEGFR-2 is followed by the activation of Src-family tyrosine kinases and CrkII, which are important for membrane ruffling and cell motility. In addition, transactivation of VEGFR-2 results in the activation of protein kinase B (PKB)/Akt (which is regulated by PI3Kα), which also phosphorylates and activates eNOS[70] (FIG. 6). What makes this interplay even more complex is the finding that S1P-stimulated PKB/Akt can bind $S1P_1$ and phosphorylate its third intracellular loop at threonine 236, which is required for Rac activation and chemotaxis that is induced by S1P[74].

Implications for cell growth. Recent studies indicate that VEGF can also stimulate SphK1, and this has ramifications for its mitogenicity[28]. It has long been known that VEGF stimulates endothelial cell growth through protein kinase C (PKC), which leads to the activation of ERK1/2. However, the exact mechanism by which this happens remained elusive until a recent study showed that SphK1 was the missing link between PKC and ERK1/2 (REF. 28). Pharmacological inhibitors, DOMINANT-NEGATIVE SphK1, or SMALL INTERFERING RNA (siRNA) targeted to SphK1 all blocked VEGF-induced, but not epidermal growth factor (EGF)-induced, ERK1/2 activation and DNA synthesis. Incubation of cells with siRNA that specifically targeted SphK1, but not SphK2, blocked the VEGF-induced accumulation of active, GTP-bound Ras. In this study, it was proposed that membrane-associated sphingosine in cells attenuates basal Ras activity by stimulating the activity of Ras GTPase-activating proteins (RasGAPs). VEGF stimulation of PKC and the consequent activation of SphK1 results in the conversion of sphingosine to S1P, which then displaces sphingosine from GAPs (FIG. 6). Overall, this would decrease GAP activity and increase the level of activated Ras–GTP, without influencing the opposing activity of Ras guanine nucleotide exchange factors (RasGEFs)[28], leading to activation of the ERK/mitogen-activated protein kinase (MAPK) pathway and cell division (FIG. 6).

An important question is whether the S1P that is produced by VEGF-mediated stimulation of cellular SphK1 activates S1P receptors to activate Ras and ERK/MAPK, or whether it functions as an intracellular second messenger. Several lines of evidence have been proposed in support of the latter possibility[28].

DOMINANT-NEGATIVE
A defective protein that retains interaction abilities and so distorts or competes with normal proteins.

SMALL INTERFERING RNA (siRNA). Short (21–23mers) sequences of double-stranded RNA that are used in RNA interference, a process by which the expression of homologous genes is silenced through degradation of their cognate mRNA.

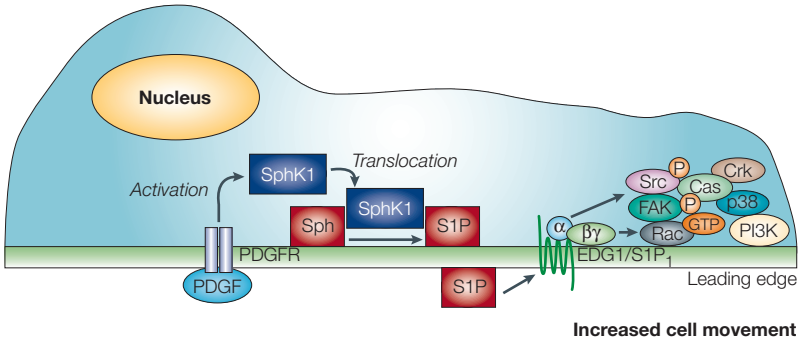


Figure 7 | **Transactivation of EDG1/S1P$_1$ and PDGF-directed cell movement.** The sphingosine-1-phosphate (S1P) kinase SphK1 is diffusely distributed in the cytosol of unstimulated cells, but platelet-derived growth factor (PDGF) rapidly induces its activation and translocation to membrane ruffles, where it phosphorylates membrane-associated sphingosine (Sph) to form S1P. S1P binds to the receptor EDG1/S1P$_1$ leading to activation of several downstream signals, such as focal adhesion kinase (FAK), Src, p38, Rac, phosphatidylinositol 3-kinase (PI3K), Cas and Crk, that are important for cell locomotion. As explained in the text, this crosstalk has ramifications for the migration of pericytes and smooth muscle cells around newly formed blood vessels.

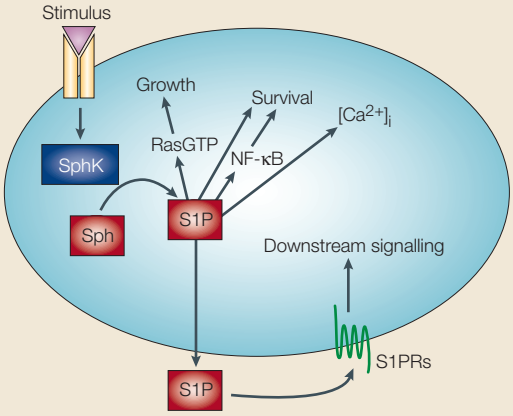
First, PERTUSSIS TOXIN blocks S1P-induced, but not VEGF-induced, ERK1/2 activation. Second, S1P–S1P-receptor activation of ERK is blocked by dominant-negative Ras, whereas VEGF activation of ERK/MAPK is insensitive. Finally, no secretion of S1P could be detected. So, it seems that signalling of VEGF and of extracellular S1P are distinct, which indicates that the intracellular S1P generated by VEGF leads to activation of downstream signalling without engaging S1P receptors[28].

Implications for movement. Reciprocal mechanisms of transactivation have been reported to be important in cell movement that is directed by platelet-derived growth factor (PDGF)[64]. Ligation of the PDGF tyrosine kinase receptor by PDGF activates and translocates SphK1 to the plasma membrane. This gives rise to the spatially restricted formation of S1P, and, in turn, activates S1P$_1$, which is essential for PDGF-directed cell movement (FIG. 7). This indicates that intracellularly generated S1P might signal 'inside–out' through its cell-surface receptors (BOX 3). S1P receptors are linked to the G-protein-mediated activation of downstream signals, such as Rac and p38, to regulate cytoskeletal rearrangements and focal adhesion turnover that is modulated by the tyrosine kinases focal adhesion kinase (FAK) and Src, which are important for cell locomotion[75] (FIG. 7). However, it is still not clear whether such inside–out signalling contributes to the effects of S1P on cell growth and suppression of apoptosis (BOX 3).

How do external stimuli stimulate SphK1?

Several recent studies have investigated the mechanisms regulating the activation of SphK1 by various stimuli, where this occurs in the cell, and whether it leads to the release of S1P extracellularly. As SphK1 is predominantly a cytosolic enzyme, whereas its substrate sphingosine is generated in membranes, it is not surprising that translocation to membranes is a common feature of SphK1 activation. For example, SphK1 is translocated to the leading edge of cells during PDGF-induced cell

Box 3 | **S1P signalling inside and out**



Diverse external stimuli, particularly growth and survival factors and chemoattractants — including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), tumour necrosis factor-α (TNF-α), nerve growth factor, epidermal growth factor, basic fibroblast growth factor, IgE/antigen, phorbol ester, vitamin D$_3$, ATP, fMLP, oxidized low density lipoprotein, bradykinin, endothelin-1, cyclic AMP, acetylcholine, lysophosphatidic acid, prosaposin and even sphingosine-1-phosphate (S1P) itself through S1P receptors (S1PRs; for a review, see REF. 22) — stimulate the S1P kinase SphK1 to generate intracellular S1P. S1P might have dual functions. First, it can function in an autocrine or paracrine fashion to stimulate S1P receptors that are present on the cell surface of the same or nearby cells. Coupling of S1P receptors to diverse G proteins leads to activation of numerous downstream signalling pathways (FIG. 4). This type of crosstalk has been shown for the PDGF receptor, which transactivates the S1P receptor EDG1/S1P$_1$ leading to the modulation of components of downstream pathways, including Src, focal adhesion kinase, and Rac, which are important for PDGF-directed cell migration[76]. It is not known whether other components of signalling pathways, such as phospholipase C (which regulates calcium levels), or phosphatidylinositol 3-kinase (which activates protein kinase B/Akt), might also be regulated in this manner. It is not even clear how S1P is secreted and presented to S1P receptors. Second, S1P might also have intracellular functions to regulate calcium levels, survival and growth. For example, crosslinking of the antigen receptor FcεRI stimulates SphK1 and S1P production, which then mobilizes calcium from internal stores independently of inositol trisphosphate formation[44,46]. TNF-α and other cytokines stimulate SphK1 leading to the activation of the transcription factor nuclear factor κB (NF-κB), which is essential for the prevention of apoptosis[30]. The potent angiogenic factor VEGF stimulates SphK1 to produce S1P, which mediates VEGF-induced activation of Ras and consequently, extracellular-signal regulated kinase/mitogen-activated protein kinase signalling and cell growth[28].

PERTUSSIS TOXIN
A mixture of proteins that is produced by *Bordetella pertussis*. It causes the persistent activation of G_i proteins by catalysing the ADP-ribosylation of the α-subunit.

PHORBOL ESTERS
Polycyclic esters that are isolated from croton oil. The most common is phorbol myristoyl acetate (PMA, also known as 12,13-tetradecanoyl phorbol acetate or TPA). They are potent co-carcinogens or tumour promoters because they mimic diacylglycerol, thereby irreversibly activating protein kinase C.

PHOX HOMOLOGY DOMAIN
A domain that is similar in function to pleckstrin homology domains. It has an affinity for certain phosphorylated phospholipids.

migration[76]. In addition, activation of PKC by the PHORBOL ESTER phorbol 12-myristate 13-acetate or VEGF phosphorylates and activates SphK1. However, whereas translocation of SphK1 to the plasma membrane was accompanied by increased secretion of S1P, which allows for autocrine/paracrine signalling in response to phorbol ester[77], no secretion of S1P was induced by VEGF[28]. In another type of activation, an elegant study identified a tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2)-binding motif of SphK1 that mediated the interaction between TRAF2 and SphK. This resulted in the activation of SphK1, which in turn was required for TRAF2-mediated activation of NF-κB and the anti-apoptotic effect of TNF[30]. Furthermore, two other unrelated SphK1-interacting proteins have recently been identified by yeast two hybrid screens. One has some similarity to a family of protein kinase A anchor proteins and affects the activity of SphK1 (REF. 78). The other, RPK118 (for ribosomal S6 kinase-like protein with two pseudo kinase domains), which also contains a PHOX HOMOLOGY DOMAIN and specifically binds to phosphatidylinositol 3-phosphate, induced the translocation of SphK1 to early endosomes[79]. It is tempting to speculate that this translocation might allow it to phosphorylate sphingosine that is produced in the salvage pathway (BOX 2).

New clues from plants

A new study shows that SphK is involved in both ABA-mediated inhibition of stomatal opening and the promotion of stomatal closure in *Arabidopsis thaliana*, by regulating guard-cell inward K^+ channels and anion channels[52]. Surprisingly, S1P regulates stomatal apertures and guard-cell ion-channel activities in wild-type plants, but not in the absence of the sole prototypical heterotrimeric G protein α-subunit (GPA1), which indicates that heterotrimeric G proteins might be downstream targets for S1P. Of particular interest, the putative GPCR of *A. thaliana*, GCR1, has no sequence homology to any of the conserved S1P receptors. Therefore, the S1P signal in guard cells might be transduced by the direct interaction of S1P with GPA1 or by unidentified proteins that stimulate heterotrimeric G proteins independently of GPCRs. As the intracellular targets of S1P in mammalian cells have not yet been identified, these studies in plants might provide clues to the enigmatic intracellular action of S1P in mammalian cell growth.

Conclusion and perspectives

Research on the sphingolipid metabolite S1P has expanded tremendously in the past few years. In this review, we have highlighted how S1P levels are regulated and how S1P is able to regulate so many physiological processes. Appropriately, considering its name, it is still a riddle why intracellularly generated S1P can signal 'inside–out' to regulate cell movement, but in the few examples studied so far, S1P receptors seem not to have a role in its growth and survival actions. There is no doubt that deciphering the complex interplay between S1P signalling inside and out and determining how it is transported into and out of cells will uncover many more hidden secrets. Studies in lower organisms, such as yeast, slime mould, plants, worms and flies, should provide clues to the ancient roles of S1P. Future work will unravel the mystery of the many hues of this simple lipid S1P.

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CHAPTER 163

Sphingosine-1-Phosphate Receptors

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Introduction

The endothelial differentiation gene (EDG) family of G-protein coupled receptors (GPCRs) comprises high-affinity receptors for the lysophospholipids, lysophosphatidic acid (LPA), and sphingosine-1-phosphate (S1P) [1,2]. The homologous EDG receptors are clearly divided into two classes: three that bind LPA (EDG-2/LPA_1, EDG-4/LPA_2, EDG-7/LPA_3) and five that bind S1P (EDG-1/$S1P_1$, EDG-3/$S1P_3$, EDG-5/$S1P_2$, EDG-6/$S1P_4$, EDG-8/$S1P_5$). Molecular modeling and targeted mutagenesis have shown that S1PRs and LPARs use very similar motifs for binding of ligands, with one amino acid primarily determining the difference in specificity [3]. As several excellent reviews have recently appeared on LPARs and our studies have concentrated on dissecting molecular signaling pathways regulated by S1P [4,5], we have focused in this chapter on lipid signaling to and through S1PRs.

S1P is formed by sphingosine kinase (SphK), of which there are two known mammalian isoforms (for review, see [6]). SphKs are evolutionarily conserved and catalyze the ATP-dependent phosphorylation of the primary hydroxyl of sphingosine, the common backbone of mammalian sphingolipids. S1P is an interesting molecule that is an intercellular messenger and an intracellular second messenger [7]. This greatly complicates interpretation of results when adding exogenous S1P to cells: Is the response observed due to cell surface receptors, effects on intracellular targets, or both? A preponderance of studies have indicated that many of the biological effects of S1P are mediated by specific S1PRs and the lack of confirmed intracellular targets appears to bolster these claims. However, others have suggested that certain results are better explained by receptor-independent intracellular effects of S1P. First, the well-known S1PRs typically have K_ds in the 2–30 nM range [2,8], whereas effects of S1P on growth and suppression of apoptosis usually require micromolar concentrations [9]. In addition, dihydrosphingosine-1-phosphate (dhS1P), which has the same structure as S1P but only lacks the 4,5-*trans* double bond, binds to and activates all of the S1PRs. However, dhS1P does not mimic the effects of S1P on growth and survival [10], thus suggesting that these effects are likely to be mediated by intracellular actions of S1P.

The S1PRs

S1P was identified as the natural high affinity ligand of $S1P_1$ [2], which was shown to be highly specific, only binding S1P and dhS1P [11,12]. $S1P_1$ is coupled to $G_{\alpha i}$ and $G_{\alpha o}$ [13] but not $G_{\alpha s}$, $G_{\alpha q}$, or $G_{\alpha 12/13}$ [14]. Thus, pertussis toxin, which inhibits $G_{\alpha i/o}$ proteins, is a useful tool for dissecting signaling through $S1P_1$. *$s1p_1$* deleted mice died *in utero* between E12.5 and E14.5 due to massive hemorrhaging [15]. Although vasculogenesis and angiogenesis are normal in the *$s1p_1$-/-* mice, vascular smooth muscle cells failed to completely surround and seal the vasculature, thereby leading to hemorrhage. On a cellular level, the defect was linked to an inability of $S1P_1$ null fibroblasts to migrate toward S1P, likely due to dysfunctional Rac activation, and indicated the important role of S1P/$S1P_1$ signaling in motility.

$S1P_2$ is unique in being the only one of the S1PRs with a significantly poorer affinity for dhS1P than S1P [16]. $S1P_2$ has a wide tissue distribution [17] and a K_d for S1P of 20–30 nM [11]. In addition to $G_{\alpha i/o}$, $S1P_2$ couples to $G_{\alpha q}$ and $G_{\alpha 12/13}$ [14]. $S1P_2$ has been linked to increases in cAMP levels and thus may couple weakly to $G_{\alpha s}$ in some cell types depending on the pattern of expression of both GPCRs and

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G proteins [18]. $S1P_2$ regulates diverse signaling pathways, including calcium mobilization, stimulation of NF-κB, and inhibition of Rac-dependent cell migration in certain cell types [19,20]

$S1P_2$ has also been knocked out in mice [21] and in contrast to *s1p$_1$*-/- mice, these mice have no obvious anatomical or physiological phenotypes. It is interesting that mammalian $S1P_2$ is highly homologous to the zebrafish gene *miles apart* [22]. Two inactivating mutations in *miles apart* prevent the normal migration of heart primordia, thus resulting in abnormal cardiac development. The heart precursor cells from the *miles apart* mutants migrated normally when transplanted into wild-type embryos, but wild-type cells failed to migrate in mutant embryos, a result that suggests that the zebrafish $S1P_2$ homologue is required for generating a migration-permissive environment.

$S1P_3$ was shown to be activated by S1P [23] with a K_d of 20–30 nM [11,12] and to couple to $G_{\alpha i/o}$, $G_{\alpha q}$, and $G_{\alpha 12/13}$, but not $G_{\alpha s}$ [14]. $S1P_3$-null mice have been generated and also have no obvious phenotype [24]. $S1P_3$ has been linked to many signaling pathways, including calcium mobilization, stimulation of NF-κB, and NO production [25,26].

The two remaining S1PRs have a more narrow tissue distribution. $S1P_4$ is expressed almost exclusively in lymphoid and hematopoietic cells, as well as in the lung [27]. $S1P_4$ has a K_d for S1P of 12–63 nM, as determined by different groups [28,29], and couples to $G_{\alpha i/o}$. The final S1PR, $S1P_5$, previously named EDG-8 and *nrg-1*, is expressed predominantly in the central nervous system and to a lesser extent in lymphoid tissue [8,30]. $S1P_5$ couples to $G_{\alpha i/o}$ and $G_{\alpha 12/13}$, but not to $G_{\alpha s}$ or $G_{\alpha q}$ [31] and has a K_d for S1P of 2–6 nM [8,31].

S1P Signaling via S1PRs

Intriguing questions concerning lysolipid messengers are what regulates the levels of these amphipathic molecules and how do they get to their target cells? Platelets are known to store S1P and release it upon stimulation (reviewed in [32]). HUVECs and C6 glioma cells release S1P to the extracellular milieu [33,34]. Moreover, even when S1P release from cells is below detectable limits, co-culturing cells expressing $S1P_1$ with cells producing S1P due to overexpression of SphK induced activation of $S1P_1$ on adjacent as well as distant cells, thus indicating either that vanishingly small amounts of S1P are released or that it can be transferred from one cell to another by cell–cell interactions, or both [35]. Thus, S1P can act in an autocrine and/or paracrine manner. In support of this concept, the chemoattractant PDGF recruits SphK to the plasma membrane, where S1PRs are located, and especially to structures known as lamellipodia [36]. Given the importance of lamellipodia and S1PRs in chemotaxis, this finding suggests that S1P is produced and released from the cell in a spatially restricted manner, providing cells with a sense of direction. In addition, a recent report claims that type 1 SphK is secreted from cells in a catalytically active form and may catalyze the formation of SIP at or near the plasma membrane [33]. Further studies are necessary to confirm a role for extracellular SphK.

Transactivation of S1PRs

An intriguing aspect of the *s1p$_1$*-/- phenotype is that it appears to be nearly identical to that of the PDGF-BB and PDGFR-β knockouts [15], as these embryos also die because of a vascular smooth muscle cell migration defect. Because PDGF stimulates SphK and increases S1P [10], it therefore appeared possible that $S1P_1$ and PDGF signaling pathways are linked. Indeed, embryonic fibroblasts from *s1p$_1$*-/- mice, in contrast to wild-type cells, failed to migrate toward both S1P and PDGF [35]. Moreover, enforced expression of $S1P_1$ in HEK 293 cells, which express low basal levels of $S1P_1$, increased their ability to migrate toward PDGF, and antisense ablation of $S1P_1$ significantly inhibited migration toward PDGF [36]. A specific inhibitor of SphK also blocked PDGF-induced motility. Taken together, these results suggest a transactivation pathway linking PDGF though SphK to the autocrine and/or paracrine release of S1P that then stimulates $S1P_1$ to regulate motility. Furthermore, it was independently shown that $S1P_1$ potentiated the response to PDGF in HEK 293 cells overexpressing PDGFR [37]. However, in this case, these effects appeared to be independent of SphK, and it was suggested that PDGFR and $S1P_1$ were tethered in a complex that was activated independently of S1P.

Downstream Signaling from S1PRs

Because the S1PRs are coupled to heterotrimeric G proteins, the types of signals transduced are many and varied, depending on the specific isoforms of G_α and $G_{\beta\gamma}$ that are present. Thus, signals linked to a S1PR in one cell type may not be linked in the same manner in a second cell type. For example, transfection with $S1P_1$ increases S1P-induced calcium mobilization in CHO cells [38] but not in COS-7 cells [39]. Determining which specific S1PR is involved in a particular response is difficult because most cells express multiple S1PRs. To date, S1PR specific agonists or antagonists have not been developed. Thus, to elucidate the role of a particular S1PR, either transfection of receptor negative or knockout cells or antisense approaches have been used. Given the diversity of GPCR signaling, it is not surprising that results from these experiments demonstrate that S1PRs control the major lipid-mediated signaling pathways, as discussed below.

Phospholipase C. Many of the responses linked to S1PR signaling involve increases in intracellular calcium. Generation of the second messenger inositol trisphosphate (IP_3) by activation of phospholipase C (PLC) is the major pathway leading to intracellular calcium increases. CHO cells transfected with $S1P_1$, $S1P_2$, $S1P_3$, or $S1P_4$, but not vector controls, had increased IP_3 production and calcium release in an

S1P-dependent manner [28]. In contrast, in Jurkat T cells, $S1P_2$ and $S1P_3$, but not $S1P_1$, elicited IP_3-mediated calcium responses [25]. On a more physiological level, in HUVECs, which express $S1P_1$, and to a lesser extent $S1P_3$, S1P stimulated nitric oxide (NO) production by calcium-dependent epithelial nitric oxide synthase (eNOS) [40]. NO production was blocked by the PLC inhibitor U73122, the calcium chelator BAPTA-AM, and antisense oligonucleotides to $S1P_1$ or $S1P_3$, thus demonstrating a role for both S1PRs in activation of PLC. Furthermore, fibroblasts from $S1P_3$-null mice, but not littermate controls, failed to activate PLC upon S1P addition [24].

Phospholipase D. Another important lipid second messenger is phosphatidic acid (PA), which is generated by activation of phospholipase D (PLD). Overexpression of $S1P_1$ in HEK 293 or NIH 3T3 cells did not result in activation of PLD [9]. However, in C2C12 skeletal muscle cells, S1P stimulated PLD via either $S1P_1$, $S1P_2$, or $S1P_3$ in a pertussis toxin–sensitive manner [41]. Transfection of either $S1P_1$ or $S1P_2$ in C6 glioma cells conferred S1P-dependent PLD stimulation and PA formation [42]. $S1P_3$ also induced production of PA, specifically through activation of PLD2 in CHO cells [43].

Phosphatidylinositol-3-kinase. Activation of phosphatidylinositol-3-kinase (PI3K) promotes cell survival, cytoskeletal remodeling, and vesicular trafficking [44]. PI3K also promotes activation of the protein kinase Akt in two ways: translocation of Akt to the membrane by binding phosphatidylinositol-3,4-bisphosphate and activation of phosphoinositide-dependent kinases, which phosphorylate and activate Akt (reviewed in [45]). Though the S1PR(s) involved were not identified, S1P induced chemotaxis and angiogenesis of endothelial cells both *in vivo* and *in vitro* in a PI3K- and Akt-dependent manner [46,47]. $S1P_1$ transiently transfected in COS-7 cells led to activation of Akt, which was inhibited by the PI3K inhibitor wortmannin [48]. Further work from this group implicated $G_{\beta\gamma}$ stimulation of the PI3Kβ isoforms in S1P-dependent signaling to PI3K [49]. On a more physiological level, ventricular cardiomyocyte hypertrophy induced by S1P was inhibited by both wortmannin and by $S1P_1$ antibody [50]. $S1P_1$, $S1P_2$, and $S1P_3$ transfected into CHO cells each activated PI3K in response to S1P [43,51]. It is interesting that in this system, $S1P_1$ and $S1P_3$ promoted S1P-induced chemotaxis, while $S1P_2$ inhibited it.

Sphingosine Kinase. S1P has been demonstrated to release calcium from non-IP_3 releasable microsomal stores, though the intracellular receptor(s) are unknown [52–54]. Meyer zu Heringdorf and colleagues demonstrated that HEK 293 cells endogenously expressing $S1P_1$, $S1P_2$, and $S1P_3$ mobilized calcium in response to S1P [55]. However, in these cells, PLC was not activated and there was no measurable production of IP_3. What is especially interesting, they found that S1P stimulated SphK and S1P production, and the increase in S1P levels, as well as calcium release, was reduced by inhibitors of SphK. S1P production was also completely blocked by pertussis toxin, indicating the involvement of G_i-linked GPCRs in the process. Thus, the remarkable observation was made that extracellular S1P regulates intracellular S1P formation [55].

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